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THE RESPIRATORY RESPONSES OF PRE-ADOLESCENT BOYS TO MUSCULAR ACTIVITY[1]

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This is a further study of rapidly growing pre-adolescent boys, ages 9 to 13 years, whose cardio-vascular responses we reported in an earlier article. In this paper consideration is given to the respiratory, metabolic, and pulse rate responses to graded intensities of work on the bicycle ergometer, to work pushed to fatigue, and to the recovery processes after strenuous effort.

The boys usually reported for work immediately after the close of the afternoon session of school. It was our custom to have each subject rest quietly in a chair for 15 or 20 minutes, then mount the bicycle and again rest when prepared for the experiment with mouth-piece and nose-clip in place. The control determinations of the metabolic and respiratory factors and of the pulse rate were followed for a period of 10 minutes. The respired air was collected in Douglas bags. In the series of experiments dealing with the effects of graded intensities of work, it was customary to have the subject carry the load for 3 minutes before the collection of expired air was begun. Between periods of work ample time was allowed for complete recovery.

The relation of the oxygen intake to the load of work. With adults who are accustomed to serving as subjects of experimentation the absorption of oxygen generally shows a linear relationship to the intensity of effort. Our data, as summarized in table 1, obtained from the pre-adolescent boys, fail to establish that relationship in them, partly due to an inordinate augmentation in the consumption of oxygen when the lightest load was undertaken. When overloads of work were carried the oxygen consumption of the boys was less than that of young men under the same load. This difference in the two groups gives another reason for failure to establish the linear relationship between oxygen consumption and intensity of effort.

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We have shown elsewhere (Schneider, 1931) that among young men when an over-load is undertaken the expected increase in oxygen intake often falls short. In only one of the boys was this clearly so; namely, in R. B. (see table 1) who was the weakest of the group. In this case the oxygen intake increased by 243 cc. when the load was stepped up from 1500 to 3000 ft.-lbs., and by 314 cc. when the load was raised from 3000 to 4500 ft.-lbs.; yet, when 6000 ft.-lbs. were carried, the oxygen intake was augmented only 114 cc.

Minute volume and frequency of breathing. The data for the minute volume and frequency of breathing are summarized in table 2. It has been found that the minute volume, frequency, and depth of breathing for men correlate with the amount of physical work accomplished per minute as

TABLE 1

SUBJECT	CUBIC CENTIMETERS OF OXYGEN ABSORBED PER MINUTE DURING WORK					INCREASE IN OXYGEN CONSUMPTION PER INCREASE IN LOAD			
	Rest	1,500 foot-lbs.	3,000 foot-lbs.	4,500 foot-lbs.	6,000 foot-lbs.	1,500 foot-lbs.	3,000 foot-lbs.	4,500 foot-lbs.	6,000 foot-lbs.
R. B.	243	754	997	1,311	1,425	511	243	314	114
J. B.	305	894	1,126	1,510	1,750	589	232	384	240
J. H.	298	936	1,299	1,503	1,785	638	363	204	282
H. C.	297	767	1,071	1,373	1,643	470	304	302	270

TABLE 2

SUBJECT	MINUTE-VOLUME OF BREATHING IN LITERS					FREQUENCY OF BREATHING PER MINUTE				
	Rest	1,500 foot-lbs.	3,000 foot-lbs.	4,500 foot-lbs.	6,000 foot-lbs.	Rest	1,500 foot-lbs.	3,000 foot-lbs.	4,500 foot-lbs.	6,000 foot-lbs.
R. B.	6.8	16.57	22.10	31.03	34.98	13	26	26	33	33
J. B.	9.9	20.32	26.04	37.19	43.67	16	30	37	39	43
J. H.	7.7	21.98	32.76	41.79	51.10	19	33	42	43	43
H. C.	7.8	20.03	25.63	34.30	42.14	13	30	29	32	36

long as the load is moderate. It will be observed that our boys failed to conform to these linear relationships. During rest the volume and frequency of breathing of boys are not noticeably different from those of men. During physical work, however, the boys breathe more frequently but inspire less air per minute than men.

Tidal air. Here again when at rest the boys do not differ materially from young men. For the boys the resting tidal air as they sat on the bicycle ergometer ranged from 403 to 675 cc. During work, however, there is a well defined difference in the depth of breathing of the two ages, which shows up under all loads of work. Thus with a load of 6000 ft.-lbs. the tidal air per breath of the boys ranged between 1032 and 1190 cc., while that of men ranged from 1378 to 2891 cc.

The respiratory dead space. For 3 of the boys during rest the dead space was 128, 152, and 158 cc., respectively. For adults it is said to range between 100 and 175 cc. Henderson finds it is about 150 cc. Normally it increases during physical effort. For our boys the following increases were typical: J. B. at rest 152 cc., with a load of 3000 ft.-lbs. 161 cc. and with a load of 4500 ft.-lbs. 190 cc. The increases in the dead space shown by the boys when at work are somewhat smaller than occur in men when the minute volume of breathing is augmented to the same degree.

The ventilation equivalent for oxygen. This has been defined by Knipping and Moncrieff as the volume of air which has to be inspired for each 100 cc. of oxygen absorbed. They found that its value for normal adults is approximately 2.4 liters and that under experimental conditions it remains unaltered after taking food or moderate exercise. Among our boys when at rest the ventilation equivalent ranged from 2.6 to 3.3 liters, which was somewhat above the normal value found by Knipping and Moncrieff. By light work, 1500 ft.-lbs., the V.E. was lowered, but as the load of work was augmented it rose slightly. As there was no instance of a large increase in the V.E., it may be assumed that in no case, even with a load of 6000 ft.-lbs., was there evidence of a large discharge of lactic acid into the blood stream, in that such a discharge should so stimulate the respiratory center that the volume of breathing would rise without a comparable increase in oxygen consumption.

Respiratory quotient. The R.Q. of rest gives no indication that any boy was overbreathing. There is nothing exceptional or unusual in the exertion records. The R.Q. rises with the metabolic rate, as it does for adults, as shown by Douglas, Haldane, Henderson, and Schneider and other workers.

Alveolar air. Sampling of the alveolar air with the subject at rest was done by the Haldane-Priestley method and during exertion by a modification of the Henderson-Haggard method. All data on alveolar air here recorded are supported by several experiments made under identical conditions. The average resting values for the boys were as follows: CO_2 in mm. Hg. R. B. 40.3, J. B. 38.6, and J. H. 38.8; O_2 in mm. Hg R. B. 101.2, J. B. 100.5, and J. H. 99.3.

Physical exertion invariably caused the alveolar oxygen pressure to rise. The data for J. B. are the most extensive. His alveolar oxygen with a load of 3000 ft.-lbs. advanced from an average of 100.5 mm. during rest to an average of 104.5 mm.; with a load of 4500 ft.-lbs., to an average of 106.4 mm.; and with a load of 6000 ft.-lbs., to an average of 108.9 mm. For J. H. with a load of 3000 ft.-lbs., the oxygen pressure rose from a resting value of 99.3 mm. to an average of 108.1 mm.; and with a load of 4500 ft.-lbs., to 111 mm. In young men at sea-level Dill and collaborators found that the alveolar oxygen pressure during physical exertion does not

necessarily change and that if it does the direction cannot be predicted; that is, it may either rise or fall, but during exertion at an altitude of 10,000 feet they found the alveolar response always showed a rise in oxygen pressure. Their maximum rise was 13.8 mm. The response of our boys, even at sea-level, was always a positive one similar to the anoxic experience at high altitudes.

Oxygen debt. The oxygen debt determined after 5 minutes of work was never clearly larger than has been obtained with young men. The boys, however, as they grew in size went less into debt for oxygen. After a period of 6 months J. B.'s debt after carrying a load of 6000 ft.-lbs. for 5 minutes was reduced from 1210 to 870 cc. and that of J. H. from 1085 to 910 cc. The growth increase in strength and possibly to some extent the factor of training account for the smaller oxygen debts. The debt was always paid promptly; by J. B. in 4 minutes, by R. B. in 7 minutes, and by J. H. between the 6th and 11th minutes.

TABLE 3

Two series of observations on R. B. made one year and four months apart

LOAD OF WORK	FIRST SERIES				SECOND SERIES			
	Respiratory minute-volume	Oxygen consumption per minute	Pulse rate per minute	Oxygen pulse	Respiratory minute-volume	Oxygen consumption per minute	Pulse rate per minute	Oxygen pulse
ft.-lbs.	*liters*	*cc.*		*cc.*	*liters*	*cc.*		*cc.*
Rest	6.8	243	107	2.3	8.2	305	86	3.5
1,500	16.6	754	141	5.3	21.1	900	132	6.8
3,000	22.1	997	165	6.4	27.4	1,200	146	8.2
6,000	35.0	1,425	197	7.2	50.1	1,860	164	11.3

Growth differences. R. B. grew rapidly while under observation. In November 1932 he was 5 feet and 3 inches tall and weighed 87 pounds. Early in April 1934 he was 5 feet 9 inches tall and weighed 116 pounds. Some comparisons of his response to physical exertion at the two periods have been tabulated in table 3. His resting intake of oxygen rose in the year and 4 months as much as 62 cc., and during work a larger delivery of oxygen to the active muscles was made for each load of work.[4] For a load of 6000 ft.-lbs. the intake of oxygen was increased 435 cc. per minute. His respiratory minute volume also became larger during rest and under exertion. The most notable difference occurred when the load of 6000 ft.-lbs. was carried; the breathing at the earlier age was only 35 liters and at latter age, 50.1 liters per minute. The reactions of the heart, as shown by the pulse rate, were more favorable during the second series of experiments. The pulse frequency was less during both rest and physical exertion. Here again the greatest difference was found for the load of 6000 ft.-lbs., with the pulse rate 197 at the earlier age and only 164 at the latter age. During

the first series of experiments R. B.'s oxygen pulse was at all times less than in the other boys, but at the time of the last series it had augmented so that it was almost up to the level found among young men. Schneider found that the oxygen pulse for young men, when carrying a load of 6000 ft.-lbs., ranged between 11.7 and 12.8 cc. R. B. in the second series of experiments with a load of 6000 ft.-lbs. had an oxygen pulse of 11.3 cc. We have shown in an earlier paper that the output of blood by the heart per beat by our boys was less than that of adults. The oxygen pulse data for R. B. suggest that his output of blood per beat was still somewhat less than that of adults, but that in a year and 4 months his heart had definitely increased its stroke volume.

Steady state. In these experiments the work proceeded until the subject felt he could continue no longer. It is generally admitted that a steady state is reached when the oxygen demand is adequately met. A steady state implies a relatively constant total ventilation, respiratory rate, absorption of oxygen, discharge of carbon dioxide, pulse rate, and internal environment.

With the load of 4500 ft.-lbs. each of the boys attained an excellent degree of steadiness in the various respiratory and metabolic responses, even though they were unable to continue the work for the same length of time. In none of them did the respiratory and metabolic data account for the fatigue that terminated the work period. That heart fatigue may have played a part in endurance is suggested by the high rates of J. B. and R. B., but this does not seem a probable explanation for J. H. whose pulse rate steadily rose but reached only 164 beats. R. B.'s pulse rate reached 210 beats the last minute of work.

A summary of the data for two of the boys while carrying a load of 6000 ft.-lbs. is given in table 4. It is evident that here again, all the respiratory responses reached a steady state while the pulse rate failed to do so. Since the heart rate alone increases progressively throughout the period of work it may be concluded that the fatigue of these boys was in large measure associated with inability of the circulation to meet the demands of the active muscles.

A striking feature in all the experiments on the steady state was that the respiratory quotient, after the early minutes of work, came back to the pre-exercise level. This indicates that during work the body continued to oxidize the same foods that it was using during rest before work began.

Recovery from a load of 6000 ft.-lbs. Each boy was asked to carry this load as long as earlier experiments had indicated would be near his limit of endurance; hence R. B. worked for 5 minutes, J. H. for 10 minutes, and J. B. for 15 minutes. If we assume, as claimed by Solandt and Ridout, that the respiratory metabolic changes are the last to disappear during recovery then these boys made an extremely rapid recovery. This might

be interpreted to mean that the boys were in excellent training. Liebenow found that as five subjects trained their recovery rate diminished after a standard exertion. Our boys, like most American boys, were fond of outdoor play and were therefore active during after-school hours; but none of them indulged in regular strenuous activity. We shall show, however, that so far as boys are concerned, it is a mistake to assume that the respiratory metabolism is the last change to disappear in the recovery process. We find when metabolism has returned to the pre-exercise level that the frequency of heart beat is still above its resting level.

A typical record of recovery is given for J. B. in table 5. In this experiment J. B. contracted an oxygen debt of 870 cc. as he carried a load of 6000 ft.-lbs. for 15 minutes. In another experiment with the same load in

TABLE 4

MINUTE	RESPIRATION RATE	RESPIRATION—MINUTE-VOLUME	OXYGEN ABSORBED PER MINUTE	RESPIRATORY QUOTIENT	PULSE RATE	OXYGEN PULSE	VENTILATION EQUIVALENT
6,000 ft.-lbs. of work per minute by J. B.							
		liters	*cc.*			*cc.*	*liters*
Rest	17	9.9	324	0.88	96	3.5	3.1
1st	30	17.8	925	0.72	150	6.2	1.9
2nd	34	32.3	1,459	0.88	172	8.5	2.2
4th	37	35.3	1,554	0.90	184	8.4	2.3
7th	40	39.7	1,645	0.90	190	8.7	2.4
10th	42	39.9	1,764	0.85	192	9.2	2.3
13th	41	38.2	1,667	0.87	198	8.4	2.3
6,000 ft.-lbs. of work per minute by R. B.							
Rest	15	5.2	243	0.78	90	2.4	2.1
1st	25	22.8	1,156	0.80	154	7.5	2.0
3rd	33	39.8	1,574	1.00	208	7.6	2.5
5th	36	39.9	1,586	0.93	224	7.1	2.5

which the steady state was studied, the "lag" in the absorption of oxygen until the maximum intake was obtained indicated an oxygen debt of 1010 cc. It appears, therefore, that in the experiment recorded in table 5 J. B. reached and maintained a steady state. Judging from other experiments we estimate that his intake of oxygen during the last minute of work was approximately 1660 cc. This fell to 1021 cc. for the first minute after work and continued to fall about as rapidly during the second minute and then more slowly during the next two minutes, after which it was back to the pre-exercise level where it remained fairly constant during the next 18 minutes of observation.

The other two boys showed much the same oxygen recovery. J. H. with a pre-exercise resting intake of 308 cc. was back to 298 cc. by the

11th minute and maintained this level for the next 8 minutes of observation. R. B. made a complete recovery in 7 minutes.

The recovery in oxygen consumption by our boys corresponded in time to that observed by Marsh and Murlin in boys 14 to 16 years of age, when they carried a load of 2170 ft.-lbs. for from 5 to 20 minutes. Their recovery required 7 or 8 minutes. Marsh and Murlin, on the other hand, found that when they prolonged the period of observation of recovery as much as 15 minutes there was an apparent decreased absorption of oxygen. There is a divergence of opinion regarding the after effect. After vigorous exercise of short duration Sargent finds that recovery in men is extremely rapid, especially in the 10 minutes immediately after exercise; but that the consumption of oxygen remains above the pre-exercise resting level for some time. Hebestreit likewise found a maintained higher resting level of oxygen intake after exercise, which he attributes to a secondary stimulus of metabolism. Marsh in a study of 19 Olympic wrestlers found, after severe

TABLE 5

Recovery of J. B. after carrying a load of 6,000 ft.-lbs. for 15 minutes

TIME	RESPIRATION RATE	RESPIRATION—MINUTE-VOLUME	CO_2 OUTPUT PER MINUTE	O_2 INTAKE PER MINUTE	RESPIRATORY QUOTIENT	VENTILATION EQUIVALENT	TIDAL AIR	PULSE RATE	OXYGEN PULSE
minutes		*liters*	*cc.*	*cc.*			*cc.*		
Rest	13	6.90	232	287	0.81	2.4	460	88	3.3
1	29	24.95	938	1,021	0.92	2.4	860	142	7.2
2	25	12.25	383	392	0.98	3.1	490	115	3.4
3–4	16	9.13	278	303	0.92	3.0	564	106	2.9
5–7	16.5	6.97	221	270	0.82	2.6	422	106	2.6
8–12	12.5	7.38	242	290	0.83	2.5	600	102	2.8
13–23	13	6.60	213	279	0.76	2.4	504	96	2.9

exertion, that the oxygen intake is depressed below normal for a period of 2 to 18 hours. Jahn, on the other hand, finds that the oxygen consumption in some cases rises, in others remains fairly steady, and in others falls.

The output of carbon dioxide per minute, as was to be expected, became somewhat subnormal as soon as the breathing began to subside. In J. B. this occurred after the 4th minute, in R. B. after the 6th, and in J. H. after the 12th minute. Later the depression in the carbon dioxide output tended to recover. For R. B., whose pre-exercise output of carbon dioxide was 191 cc., the low period during recovery extended from the 7th through the 13th minutes when it averaged 165 cc.; while from the 14th through the 19th minutes the average output was 181 cc. The gas analysis of expired air showed clearly this same swing in carbon dioxide output. For J. B. the percentage of expired CO_2 during the control rest period was 3.67, by the 3rd minute after exercise it was lowered to 3.32, for the next 4 minutes it averaged 3.45, and for the 8th through the 13th minutes it

rose to an average of 3.57. The other boys reacted similarly. The evidence is clear in two of our boys that depression in the output of CO_2 during recovery was of short duration and probably in no instance exceeded 20 or 30 minutes.

The respiratory quotient was, as is usual after strenuous exertion, high for several minutes. It then slowly fell to normal or even subnormal as contrasted with that of the control period that preceded exercise. J. H. had an R.Q. of 0.79 before work and of 0.78 during the 12th through the 20th minutes of recovery. R. B. whose R.Q. during the control period was 0.78 showed the low level of 0.74 from the 7th to the 20th minutes of the recovery study. J. B. (table 5) developed the R.Q. depression after the 13th minute and had an average of 0.76 for the next ten minutes, after which observations were discontinued. Marsh and Murlin invariably found that the R.Q. was lowered by exercise.

The minute-volume of breathing during the steady state for the load of 6000 ft.-lbs. averaged about 40 liters. It fell in all the boys during the first minute of recovery to between 20 and 25 liters and was back to the pre-exercise level within from 5 to 11 minutes. In 2 of the boys there was a period when it was slightly subnormal; in R. B., with a pre-exercise minute-volume of 5.63 liters, the average during the 7th to 13th minutes of recovery was 5.25 liters and the average during the 14th through the 19th minutes rose to 5.52 liters.

The ventilation equivalent for all three of the boys indicates that the respiratory metabolic balance was quite completely restored within the time we studied the recovery. Early in the period of recovery the minute-volume of breathing is distinctly out of proportion to the amount of oxygen that is consumed. The disproportion is slowly redressed, but eventually the pre-exercise proportion is fully restored. Thus the V.E. of J. B. (table 5) preceding exercise was 2.4, it rose to 3.1 the second minute of recovery and then slowly during the next 11 minutes returned to the pre-exercise level and there remained during the following 10 minutes of further observation. The V.E. for the three boys was fully restored to normal within from 7 to 13 minutes.

The recovery in the frequency of breathing was the most prompt of all the respiratory factors observed. In the case of J. H. the recovery was complete by the 3rd minute, in R. B. during the 4th minute, and in J. B. during the 8th minute. No depression in the frequency below that of the control period was observed.

The recovery of the 6 respiratory factors studied by us was completed first by the frequency of breathing, next in order came the usage of oxygen, then the ventilation equivalent, this was followed by the respiratory minute-volume, and lastly by the carbon dioxide equilibrium. The respiratory quotient in two of the boys was still depressed when observa-

tions were stopped. Hebestreit in a study of adults found that the ventilation first attains the resting state, followed by oxygen consumption and lastly by the carbon dioxide elimination.

A study of the frequency of the heart beat and the oxygen pulse brings out certain interesting facts regarding the recovery from the effects of strenuous physical activity. During the last minute of the work period under the load of 6000 ft.-lbs. the pulse rate was 192 for J. B., 196 for J. H., and 204 for R. B. In none of the boys was the pre-exercise rate restored in the period of observation. The pre-exercise rates for J. B., J. H., and R. B. were 88, 96 and 95; and at the end of the period of observation of 20 to 23 minutes the rates were 96, 114 and 106, respectively.

The oxygen pulse during and immediately after exertion is much larger than it is during rest. In our boys, after carrying the 6000 ft.-lb. load, the oxygen pulse was subnormal by the end of the second minute in each. The reaction of J. B., as shown in table 5, was similar to that of R. B., each reaching the lowest value between the 5th and 8th minutes. After this each made some recovery, but the oxygen pulse was still subnormal at the close of the period of observation. In J. H. the oxygen pulse reached the lowest value during the 12th minute and remained at that point during the period of observation. There are possibly two interpretations of the changes in the oxygen pulse during the period of recovery; these may be indicative either of variations in stroke volume of the heart or of variations in the unloading of oxygen from the blood as it flows through the tissue capillaries. The former is probably the interpretation that applies to our data. From this fact, and from the delayed return of the pulse rate, it may be concluded that the effects of exertion were more profound on the circulation than on respiration and metabolism and that recovery occurs more slowly in the circulatory than in the respiratory factors.

SUMMARY

A linear relationship between the consumption of oxygen and load of work was not in evidence. With light loads the intake of oxygen was too large and with heavy loads too small.

The resting rate of consumption of oxygen increased with the growth of two boys and at the same time the delivery of oxygen during strenuous work was augmented.

During rest the minute-volume, frequency, and depth of breathing of pre-adolescent boys correspond to that of adults.

The amount of air breathed per minute and the depth of breathing during exertion are smaller among boys than among adults, while the frequency of breathing is greater among boys.

The respiratory dead space during physical exertion increases less in boys than in adults.

The ventilation equivalent for oxygen is somewhat larger for boys than for men.

During physical exertion the pulmonary alveolar oxygen pressure invariably rose, the rise ranging upwards to 12 mm. Hg. Among men the alveolar oxygen pressure ordinarily shows no regular change.

The oxygen debt was never large. After six months of growth the debt with a heavy load was reduced.

Growth resulted in more favorable oxygen intake, in a larger lung ventilation, in a slower pulse rate, and in a larger oxygen pulse, both during rest and exertion.

In work carried to fatigue a steady state was ordinarily reached in all respiratory and metabolic factors; but the pulse rate, while maintaining a fairly steady state for a while, always showed some further accelerations with the onset of fatigue.

In recovery the metabolism returned to the pre-exercise level before the pulse rate. The slow return of the pulse rate and oxygen pulse indicates that strenuous exertion more profoundly disturbs the circulation than it does respiration and metabolism.

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AN EXPERIMENTAL ANALYSIS OF COAGULANT ACTIVATION

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In view of *a*, the demonstrable existence (questioned by some authorities, 23) of small quantities of antithrombin (15) and heparin (17) in normal blood plasma, *b*, their marked anticoagulant effects in vitro and in vivo, Howell (12–18) has insisted on a coagulation theory in which the phospholipids are denied any other rôle than that of neutralizing the antithrombic agents. The contrary viewpoint, extant since Morawitz (22), has especially been supported by Bordet (1, 2) and by Mills (19–21). The last named author made special efforts to establish the fact that calcium ions alone were insufficient to activate prothrombin, cephalin being equally necessary. Since the experimental evidence was difficult to obtain and, as far as we are aware, has not been conclusively confirmed, we have been impelled to re-study the problems involved, with an especially careful approach to control of technique. The enquiry has been extended to include an experimental analysis of the following coagulants: 1, plasma prothrombin; 2, platelets; 3, corneal-, and 4, crystalline lens extracts. The eye tissues were chosen because they are *physiologically* bloodless and hence avoid the criticism applicable to other tissue coagulants, viz., the possibility that part, at least, of their clotting powers may be due to the gross blood therein contained (24).

Experimental technique. Anesthetised dogs were bled into $\frac{1}{10}$ volume of isotonic trisodium citrate solution (3.8 per cent, 11) via a paraffined cannula in the femoral artery. The clear plasma obtained after repeated centrifugation was *rapidly* aspirated through a sterile Berkefeld "V" or "N" filter. Goddard's (10) denial of Cramer and Pringle's (4) contention that such "deplateletization" abolished plasma coagulability was abundantly confirmed.

Prothrombin-free fibrinogen was prepared from the above plasma by $Mg(OH)_2$ adsorption followed by at least two "saltings" with ammonium sulphate and a final precipitation with sodium chloride. The purified fibrinogen dissolved readily in distilled water and gave very rapid and firm clots on the addition of active thrombin. The ability of the fibrinogen solution to withstand cephalin plus calcium for 24 hours at 38°C. without clotting was a rigorous proof (Mills') of the complete absence of prothrombin.

Prothrombin was prepared from defibrinated (54°C.) Berkefeld plasma by Howell's acetone method (17, 3). These prothrombin solutions showed an unexpected stabil-

ity in that they could be kept for several days in the ice-chest with but little loss of potency. Fresh solutions, however, were used in all the cited experiments.

Platelets, carefully separated from corpuscles by differential centrifugation, were washed repeatedly with isotonic sodium citrate and freshly suspended in distilled water or 0.9 per cent NaCl solution.

The clear *cornea*, and also the *crystalline lens*, were dissected from the dogs' eyes freshly post-mortem. After rinsing in saline the tissues were dried rapidly in an air current at room temperature and stored in clean dry bottles. Extracts were made some hours before use by macerating in sterile salt solution.

Boiled extracts were obtained by boiling the various solutions for one minute. *Benzene extractions* were carried out by simple shaking in a separatory funnel with 3 to 5 volumes of cold benzene (6, 20).

Cephalin was prepared from calf brain by an extended process of purification aimed to secure freedom from *a*, proteins; *b*, fats; *c*, cholesterol; *d*, glycolipids, and *e*, lecithins. The acetone-insoluble material was finally purified by repeated (cold) absolute alcohol precipitation from ethereal solution. The pure white deposit from the final precipitation was preserved under a large volume of absolute alcohol (which protected it from oxidation). Just before use a small quantity of the alcoholic suspension (sediment) was evaporated to dryness in a tared flask over a boiling water bath. The residue was resuspended in distilled water. *A 1:2,000,000 (final) dilution of a 3-months old cephalin speeded up the clotting of fibrinogen by Ca-prothrombin from 8½ minutes to 1 minute.*

Coagulation times were determined in 8 mm. coagulation test tubes immersed in a water bath thermostatically kept at body temperature (38°C.). Since interest centered in the activation of coagulant rather than the completion of clotting (the tubes were usually invertible in a relatively short time later), the recorded times denote commencement of coagulation. In order to facilitate comparisons, measured quantities were employed throughout. The dilution variable (v. infra) was minimised by permitting "activation" of coagulants for several minutes at 38°C. before adding the fibrinogen. All tubes were observed over a period of 2–3 days, although very few clots were formed after the first day and the "0" (no clot) sign in the tables refers to the 24-hour reading unless otherwise specified. With the rare exceptions noted, all clots were complete within a relatively short time from commencement. The cited results were substantiated by many hundreds of tests representing a searching investigation of the process of coagulant activation.

RESULTS. A. *The cephalin factor in the activation of prothrombin.* Cephalin + calcium, at all stages in the preparation of prothrombin-free fibrinogen, gave quicker clots than calcium alone. Traces of prothrombin, too small to produce coagulation in the presence of calcium salts only, still caused good clots when cephalin was also added. The final fibrinogen preparation failed to coagulate in 24 to 48 hours with calcium + cephalin, but it was clotted *a*, with variable tardiness (10 min.–2 hrs.) by Ca-prothrombin; *b*, speedily (5–10 min.) by calcium + cephalin + prothrombin, added separately (dilution effect), and *c*, with great rapidity (5–15 sec.) when these three reagents were permitted to interact for 5 to 15 minutes prior to the addition of the prothrombin-free fibrinogen.

Benzene-extracted prothrombin-free fibrinogen gave no clot with calcium and cephalin, alone or in combination. Ca-prothrombin caused the

appearance of an incomplete clot overnight, as compared with a 5 to 10 second clot with Ca-cephalin-prothrombin. Benzene extraction of the prothrombin, also, removed the slight capacity for activation by calcium alone. Cephalin − Ca − b.e. prothrombin gave a 30-second coagulant. Tested against ordinary (unextracted) fibrinogen, this b.e. prothrombin gave 3½ minutes' clotting when calcium salts were added, and 100 seconds' clotting when activated by cephalin + calcium.

Thrombin, prepared by the interaction of prothrombin, calcium, and cephalin, could not be inactivated by simple benzene extraction (even overnight), but still gave 30 to 40 second clots (on the following day) when tested against benzene-extracted and ordinary fibrinogens.

TABLE 1

Clotting reactions of coagulants. Dog 11. 38°C.

COAGULANT	FIBRINOGEN, IN THE PRESENCE OF					
	(a) Nil	(b) $CaCl_2$ (Cephalin 24 hrs. later)		(c) Cephalin (Ca, 16 hrs. later)		(d) Ca + cephalin
1. Prothrombin	0	1¾ hrs.		0	Few minutes (<8 min.)	45 seconds
2. Platelets*	0	Overnight (>3 hrs.)		0	1½ hrs.	Overnight (>3 hrs.)
3. Cornea*	0	20½ hrs.		0	3½ hrs.	2 hrs.
4. Lens*	0	0†	1 hr.	0	Overnight (>9 hrs.)	Overnight (>3 hrs.)
5. 0.9 per cent NaCl (control)	0	0	0	0	0	0
	A.	B.	C.	D.	E.	F.

* Weak extracts.
† Confirmed.

The loss of the ability of *benzene-extracted prothrombin* to form coagulant with calcium alone was restored not only by the addition of cephalin (v. supra) but also my means of *a*, the benzene extractives (evaporated to dryness and resuspended in distilled water), and *b*, extracts of washed platelets and eye tissues (v. infra).

Poor (*e.g., aging*) *prothrombin* preparations, inactive on simple recalcification, were often found to recover their activity in the presence of the cited reagents. The effect of cephalin was studied carefully and it was noted that the re-activation was at first slow but speeded up remarkably after 2 to 3 hours (38°C.) just before reaching the maximal degree of activation (a 3–5 min. thrombin).

Benzene extraction experiments on *citrated plasma*, before and after Berkefeld filtration, showed a progressive loss of coagulative function (on simple recalcification). These recalcified plasmas were rapidly coagulated

by cephalin or platelets and gave good but slower clots with the benzene extractives.

We have observed that the *various lipoid solvents* can act on plasma in one of two ways. Most of them (e.g., alcohol, ether, acetone) definitely

TABLE 2

Clotting reactions of benzene-extracted (B.E.) coagulants. Dog 11. 38°C.

COAGULANT * (BENZENE EXTRACTED)	BENZENE-EXTRACTED FIBRINOGEN, IN THE PRESENCE OF			
	(a) Nil	(b) $CaCl_2$ (cephalin 12 hrs. later)		(c) Ca + cephalin
1. *Plasma prothrombin	0	0	Few minutes (<5 min.)	30 seconds
2. *Platelet extract	0	0 (12 hrs.)	6¾ hrs.	Overnight (>2 hrs.)
3. *Cornea extract	0	0 (12 hrs.)	Overnight (>11 hrs.)	24–36 hrs.
4. *Lens extract	0	0 (12 hrs.)	Overnight (>11 hrs.)	24–36 hrs.
5. 0.9 per cent NaCl	0	0	0	0
	A.	B.	C.	D.

TABLE 3

Clotting reactions of boiled coagulants. Dog 11. 38°C.

REAGENT	FIBRINOGEN + $CaCl_2$		FIBRINOGEN + $CaCl_2$ + PROTHROMBIN	B.E. FIBRINOGEN + $CaCl_2$ + B.E. PROTHROMBIN
	(a) Alone	(b) 6 hrs. later, + cephalin		
1. *Boiled* prothrombin	0	Few minutes* (<5 min.)	5 minutes	Flocculent clot after 6 hours
2. *Boiled* platelets	0	Overnight (>6 hrs.)	10 seconds	15 seconds
3. *Boiled* cornea	0	2 hours	10 seconds	30 seconds
4. *Boiled* lens	0	Overnight (>6 hrs.)	25 minutes	20 minutes
5. 0.9 per cent NaCl (control)	0		1¾ hours	0
6. Cephalin (control)	0		45 seconds	30 seconds
	A.	B.	C.	D.

* Another prothrombin (boiled 1 min.) + Ca + cephalin + fibrinogen (added simultaneously)—C.T. = 55 min.

precipitated and denatured the proteins as evidenced by the gross turbidity and irreversible loss of coagulative functions. A smaller group, exemplified by benzene, yielded plasmas of but slightly increased opalescence. These plasmas kept well and recovered their coagulative functions on

adding cephalin or materials containing "available" sources of that phospholipid.

B. *An experimental analysis of coagulant activation.* Parallel experiments (table 1) were performed on 1, plasma prothrombin; 2, washed platelets; 3, corneal-, and 4, crystalline lens extracts. None of these had any coagulative (thrombic) function prior to activation. Calcium salts alone were ordinarily sufficient to convert them into coagulant, *except* in the case of the lens extract, which needed cephalin in addition. Cephalin + calcium always gave better coagulants, i.e., maximal activation. In the absence of added calcium, cephalin had no activating power even in the case of the eye extracts which might be presumed to contain some calcium salts.

Benzene extraction of the 4 coagulant precursors always removed the capacity for activation by calcium alone, but cephalin, added subsequently, restored the status quo, and even activated the b.e. lens extract. Calcium + cephalin, incubated with the precursor material for several minutes prior to the addition of fibrinogen, gave optimal results. Since ordinary fibrinogen contains relatively large amounts of "available" phospholipid (v. infra), it was imperative to perform the foregoing tests on benzene-extracted fibrinogen (table 2).

After *boiling* the reagents, calcium alone was insufficient for thrombin formation. Cephalin, in addition to calcium, however, gave potent coagulants, even in the case of the lens extract. Indeed, boiling caused a considerable increase in the activity of the thrombins obtained (table 3).

Cephalin-like action. On testing the ability of the various materials to accelerate the clotting of prothrombin-free fibrinogen by a Ca-prothrombin preparation which required $1\frac{3}{4}$ hours to cause clotting, it was found that platelets and corneal extract reduced the clotting time to less than a minute, while lens extract had also a definite effect (59 min.). With *boiled* reagents an even more striking acceleration of clotting was observed (10 sec. with platelets and cornea, 25 min. with lens extract). Almost identical values resulted on adding the respective boiled materials to a system containing benzene-extracted prothrombin and fibrinogen (which remained unclotted on simple recalcification). The resemblance to the action of cephalin is striking (table 3). The adjuvant effect of boiled prothrombin is also noted in the cited table. It is suggested that Howell's prothrombin is poor in, but not quite devoid of, "available" phospholipid. In common with the other coagulant precursors it yields a considerable increase in this factor on boiling (heat denaturation).

Discussion. *Plasma prothrombin.* The careful avoidance of contact with injured tissues during collection of the blood and the completeness of removal of the formed elements by centrifugation and ultrafiltration strongly support the view that prothrombin originates from the plasma

(globulin-) proteins. It is inconceivable, in our opinion, that it could have a cellular (e.g., platelet) origin under the conditions of the present experimentation.

Although, *as ordinarily prepared*, prothrombin yields an active coagulant in the presence of calcium alone, the further addition of cephalin greatly enhances and stabilizes the thrombic activity. In ageing preparations or when a mere trace of prothrombin is present, added cephalin, given sufficient time, may be essential for the demonstration of the ability to form coagulant. Since we may fairly assert that the absence of antithrombic agents was secured by the processes of purification, it follows that the clot-facilitating action of cephalin must represent a *direct* effect on the activation of the coagulant. The benzene extraction experiments demonstrate the successful removal of a clotting factor, detectable in the extractives, but best replaced by cephalin and tissue extracts (particularly boiled extracts). Benzene extraction of the test fibrinogen is an essential part of the technique. The data afford strong support for the conclusion that a phospholipid factor (identified with free or dissociable cephalin), which can be removed by sufficiently vigorous shaking with cold benzene, is as essential as the calcium ion in the direct activation of plasma prothrombin. The ability of thrombin, once formed, to resist the inactivating influence of benzene suggests that the cephalin enters into a firmer (? chemical) union with the prothrombin (and calcium ?) during the process of thrombin formation.

Coagulant activation. The experimental analysis of the process of activation of the 4 coagulant precursors, viz., plasma prothrombin, platelets, corneal-, and lens extract, emphasizes the underlying similarity of the process in each case. Thus, 1, all require activation; 2, added calcium salts are essential, and calcium alone suffices to activate all *untreated* preparations except lens extract; 3, cephalin, in addition to Ca greatly improves the coagulant potency in all cases, and causes clotting power to appear in *a*, lens extract, and *b*, benzene-extracted reagents. The data support the conclusions that cephalin is as necessary as calcium for the direct activation of *all* these coagulants, and, when calcium appears to act alone, it is because the materials (including the fibrinogen) contain what we may conveniently term "available" phospholipid. The evidence favors the thesis that the coagulant (thrombin), in all cases consists of protein (? pseudoglobulin), phospholipid (? cephalin) and calcium components. *Until it has been shown possible to prepare a phospholipid-free thrombin capable of producing the typical coagulation of phospholipid-free fibrinogen, with the aid of calcium alone, we must believe that thrombin is essentially a cephalin-protein (complex or) compound.*

Quantitative aspects of coagulant activation. The minor differences between the reactions of the products studied are interpreted as expressions

of variability in the proportions of the three components named. On adding the missing factor(s), a coagulant is formed in each case. By keeping one factor fixed and adding an excess of the other two, the maximal clotting effect obtainable from the fixed factor can be estimated in terms of the clotting time, since it has been shown repeatedly (5, 8) that C.T. is inversely proportional to concentration to coagulant (thrombin) formed.

In the absence of analytical data on *a*, protein; *b*, phospholipid, or *c*, calcium (without which a large variable due to *dilution* is uncontrolled), the preliminary computations of table 4 are expressed in terms of hypothetical units per cubic centimeter of the particular material with which we have been experimenting. Five-tenths cubic centimeter (measured) quantities

TABLE 4

Relative strengths of factors in various coagulants

COAGULANT	PROTEIN FACTOR (PROTHROMBIN-LIKE)	LIPID FACTOR (CEPHALIN-LIKE)	LIPID FACTOR (IN BOILED REAGENT)	CALCIUM FACTOR
1. Prothrombin (ex plasma)	44.5	0.05?	0.09?	0
2. Platelet suspension	0.37	0.05	133.3	0
3. Corneal extract	2.5	4.2	66.7	? negligible trace
4. Lens extract	0.1	0	100.0	? negligible trace
5. Fibrinogen solution	0	9.5		0
	A.	B.	C.	D.

Calculation:

$$2 \times \frac{1000}{\text{C.T. (seconds)}} = \text{"units" per cubic centimeter}$$

A. *Prothrombin factor:* Obtained on prothrombin-free fibrinogen (5) in presence of excess of cephalin + calcium.

B, C. *Lipid factor:* Obtained on benzene-extracted fibrinogen (prothrombin-free) in presence of benzene-extracted prothrombin + calcium.

D. *Calcium factor:* Obtained on fibrinogen (prothrombin-free) in presence of added prothrombin + excess of cephalin.

of coagulant precursor solution were used for each cubic centimeter of test fibrinogen. Results were calculated by means of the formula:

$$2 \times \frac{1000}{\text{C.T. (seconds)}} = \text{"units" per cubic centimeter}$$

The method is based upon that by which Fischer (8, 9) investigated thrombic activity, and, by analogy, we shall speak of *a*, prothrombin units; *b*, cephalin units, and *c*, calcium units. The clotting times (not cited) represent the best values in a large number of tests.

The preliminary quantitative data suggest, tentatively (in the absence of control of the dilution variable), that the protein factor of plasma pro-

thrombin is large enough in comparison with that of platelets, cornea, and lens material to explain its occurrence in the last three materials as the possible result of diffusion from the blood stream, via the lymph and tissue fluids in the case of the eye tissues.

Since the hypothesis of the three essential components of thrombin is supported by experiments which avoid the needless complication of oxalation whereby Mills (21) sought to differentiate between coagulants of plasma and tissue origin, the assumption of such differences is unnecessary. The complex effects of oxalate, of course, require independent elucidation (7).

It is probable that the clotting of blood normally owes its inception to irreversible changes (denaturation phenomena) in platelets, in tissue elements, and, perhaps to a greater extent than is usually admitted, in the plasma "protein complex" (23). These changes render "available" enough cephalin (phospholipid) to overcome the normal protective mechanism afforded by the antithrombic factors (heparin and antithrombin), and to enter into the formation of the thrombin complex (calcium also being necessary). We believe that the rôle of the antithrombic factors is to take care of situations, physiological and pathological, in which a sudden increase in the "available" cephalin in the blood stream threatens, since calcium and prothrombin are ever-present, to produce thrombin and the attendant risk of intravascular clotting. Under "physiological" situations may be instanced the *lipemia* following meals, the correlation of which with a phase of increased coagulability of the blood is well recognized. Of pathological conditions, *hemophilia* is the most interesting. An elucidation is suggested on the basis of an exaggerated stability of lipoproteins (sources of cephalin) retarding the liberation of the necessary cephalin. Our viewpoint may be termed a "cephalin availability theory." It admits of a definite solution of the coagulation problem along physico-chemical lines.

SUMMARY

Experiments are offered in support of an hypothesis which regards coagulants, whether of plasma or tissue origin, as protein-cephalin-(calcium) compounds. A quantitative technique is outlined for evaluating the relative strengths of these essential components of the coagulant complex.

It is tentatively suggested that the protein factor, in all cases, originates from the plasma globulin (prothrombin).

The necessity for cephalin in the direct activation of the precursor material directs attention to "available" sources of that phospholipid. The great increase in cephalin "units" after boiling is believed to indicate that the denaturation phenomena following the shedding of blood (and lysis of platelets and injured tissues) make "available" more cephalin than can be

taken care of by the antithrombic factors. Howell's theory of the rôle of the antithrombic agents is modified in favor of a *cephalin availability theory* on the basis of which an explanation of the normal in vivo incoagulability of the blood and of the clotting time changes *a*, after meals, and *b*, in hemophilia is vouchsafed.

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THE ACTION OF A SINGLE VAGAL VOLLEY ON THE HEART OF THE EEL AND THE TURTLE

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For several years I used as a class demonstration of inhibitory nerve effect, a vagus-heart preparation of the eel because of its readiness to respond to single induction shocks. Although from such records only marked inhibition could be detected, it was obvious that the inhibiting effect depends on that phase of the normal heart cycle at which the stimulus was applied. As an explanation I used the generally accepted assumption that in consequence of the arriving vagal impulses A.C. (an acetylcholine-like substance) is freed and later disappears by diffusion or destruction by an esterase. The dependence of the inhibitory effect on the beat cycle, in the case of a single vagal volley, I believed due to a high rate of A.C. formation and its rapid disappearance. It was not until the summer 1934 that I found the opportunity to study in detail this time relation of assumed A.C. formation and disappearance. The results obtained were so peculiar that I repeated the experiments the next summer, only to confirm my previous findings, which I later also found in turtles.

METHOD. Most of the experiments on eels (Anguilla and Conger) were performed with the brain and the upper part of the spinal cord pithed through a small opening in the skull, the wound having been filled with absorbent cotton to minimize blood loss. A constant flow of sea water was kept running through the gills. Such a preparation remained in good circulatory condition for several hours. In a few experiments an excised vagi-heart preparation was perfused by eel Ringer (Fischer, 1926). Both preparations reacted in the same way to vagal stimulation. In most of the experiments both vagi were stimulated simultaneously with induction shocks, the heart beats were recorded by the suspension method either from the ventricle or the auricle on superficially smoked drums rotating at a speed of 3 to 5 cm. per second.

In turtles (mostly Pseudemys), after pithing them in the same manner as the eels, a hole 3 cm. in diameter was cut with a trephine into the plastron exactly above the heart. The movements of both the auricles and of

the ventricle were recorded at the same time by the suspension method either on a superficially smoked drum or on a photo-kymograph simultaneously with the electrocardiogram taken by direct leads. The vagi were stimulated in the neck by condenser discharges; each vagus alone or both simultaneously.

EXPERIMENTAL RESULTS. *The dependence of the chronotropic effect on the rhythmic mechanism of the pacemaker.* The results in experiments on eels as well as on turtles, when the ventricular beat was recorded, could be easily divided into two classes: 1, lengthening of the first influenced ventricular cycle up to 75 per cent, and 2, lengthening of the first cycle over 100 per cent or even over 200 and 300 per cent. In experiments in which the auricular beat was used as indicator, and the contraction of the interjugular part of the sinus in eels or of the vena cava superior in turtles was well expressed in the auricular curve, it became obvious that the extremely long ventricular heart cycle was often due not only to a chronotropic effect upon the pacemaker, but mainly to a blocking of the conduction from the pacemaker to the auricles (fig. 1). Therefore, the true chronotropic effect upon the pacemaker can be detected only in those experiments in which the time relation of the beats of the pacemaker can be seen in the auricular tracing or in the electrocardiogram in experiments on turtles.

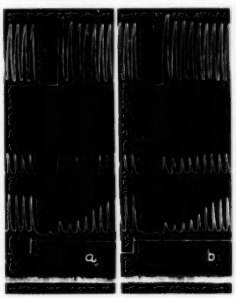

Fig. 1. Turtle. Upper curve: ventricle, then left auricle, right auricle, signal, time in $\frac{1}{5}$ second. Right vagus, condenser discharge. Stimulation in b about 1 10 cycle later than in a. 3 18 36.

Measuring carefully such records from the eel experiments, typical results were obtained as shown in figure 2. The vertical lines represent the occurrence of excitation in the pacemaker, the beginning of the jugularis movement being taken as the moment of excitation. The arrows mark the approximate moment at which the vagal impulse reaches the pacemaker. These moments have been calculated from the moments of stimulation by experimentally determined conduction times of the vagal nerves employed and by the shortest latent period obtained by direct stimulation of the pacemaker. Such a calculation, however, can only be an approximate one with an error of at least 0.05 of a cycle length; but this error is always the same throughout the whole series of stimulations in one experiment. The crosses indicate the time at which a normal beat of the vena jugularis would occur, if the cycles were not prolonged. The height of the crosses above the basal lines indicates the amount of inhibition in percentage of normal cycle length. The figures on the left of the basal lines are the times, in fractions of a normal cycle,

between the arrival of the volley and the moment at which the next normal beat would appear.

A vagal volley arriving at about the moment of a normal beat does not influence at all the cycle between this beat and the next, but has a moderate but marked effect upon the later cycles. If the volley arrives later than $\frac{1}{3}$ cycle length after the initiation of a normal beat, but earlier than $\frac{1}{4}$ cycle before the next expected beat, the cycle in progress is lengthened to a great extent and the later cycles are only very little influenced. If the volley reaches the heart later in the cycle, less than $\frac{1}{4}$ cycle before the

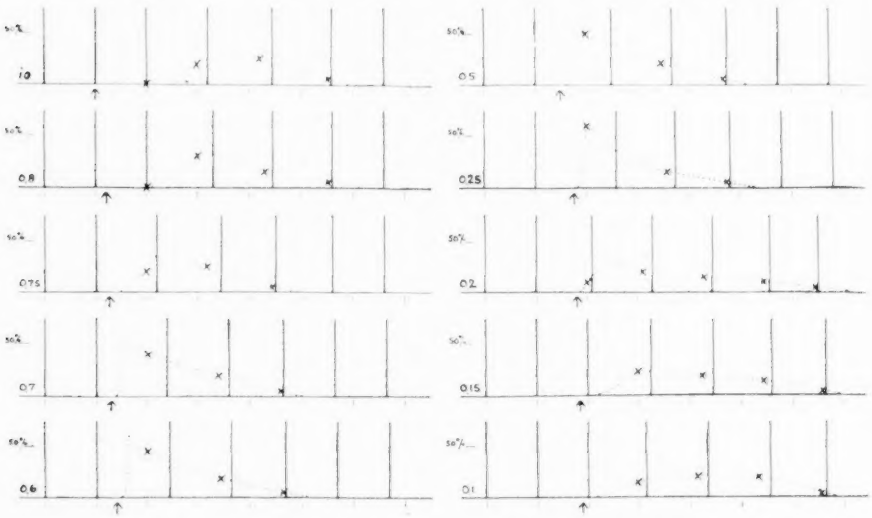

Fig. 2. Eel, both vagi stimulated simultaneously by single, just maximal condenser discharges. Vertical lines: excitation of the pacemaker. Arrows: the moments, at which the vagal impulses reach the pacemaker. Crosses: strength of inhibitory effect. Time in normal cycle lengths. For further explanation see text. 4/29/34.

next beat, the initiation of that beat is less delayed, and also the lengthening of the next cycle is less than one might expect. The later cycles are influenced to a relatively great extent.

It is impossible to draw through the experimentally found points identical curves representing the A.C. formation and disappearance for the different stimulations. Assuming that the retardation of a beat is proportional to the A.C. present in or around the pacemaker at the moment a normal beat would start, one should expect identical A.C. curves when the volleys reaching the pacemaker at various phases of the cardiac cycle would always produce, after a constant latent period, a definite amount

of A.C. at a constant rate. The hypothetical dotted curves in figure 3 are drawn in such a way as to allow a relatively simple explanation of the experimental facts.

A volley arriving about half way between two beats liberates after a short latent period a relatively large amount of A.C. at a high rate. In these cases the declining part of the A.C. curve may represent the diffusion and destruction of the A.C. When the volley reaches the pacemaker at the moment of a normal beat or shortly after, A.C. is set free only after a

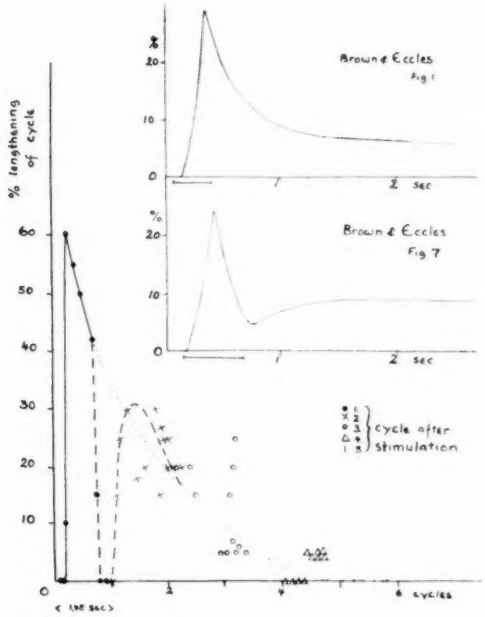

Fig. 3. Same experiment as in figure 3, all experimental points plotted in one graph, the moment of arrival of the vagal volley taken as zero time. Upper inserted curve: single wave type A.C. curve; lower inserted curve: double wave type A.C. curve, (Brown and Eccles, 1934). Further explanation in text.

very long latent period and at a much slower rate, but for a longer time, so that A.C. is still being liberated in the later part of the curve. If a volley arrives at any other time of the heart cycle, both the quick and the slow liberating mechanism come into play, each acting only in part.

Brown and Eccles (1934), who investigated the effects of single vagal volleys upon the cat heart, used another way of representing the experimental results. They plotted all data gained by stimulations at various points in the heart cycle, using the moment of stimulation as zero time

and measuring the degree to which a beat was retarded. In most of their experiments they were able to draw through their numerous points a very smooth curve, as shown in the upper inserted curve of figure 3. Representing my data in such a way (fig. 3), a similarly shaped curve can only be drawn through the very first points. The dotted part of that curve is obviously contradictory to the experimental facts. The broken line would represent the experimental findings more accurately, but its last part is indeterminable. In my experiments such a method of representation cannot give a definite A.C. curve because the formation of A.C. is not constant, but varies with the phase of the heart cycle at the moment of stimulation. Nevertheless this attempt of representation is not only useful in summarising the result of a series of stimulations, but helps—as will be discussed later (p. 604)—to demonstrate that we are not dealing with a peculiarity of the eel, but with a more general principle.

Stopping the bloodflow or the perfusion through the heart, or cutting away the ventricle, had no significant influence upon the chronotropic effect of a single vagal volley. Of all measures tried, only vagal fatigue had a distinct influence. The vagal fatigue was produced by several prolonged tetanic stimulations of both vagi and allowing a sufficiently long time to elapse for the normal heart cycle to be restored. In all experiments of this kind the dependence of the vagal effect on the heart cycle was not altered, but in most of the experiments, after fatigue, the early quick liberation process decreased in importance while the late slow one increased (fig. 4). In consequence of this shift in importance, the trough in the A.C. curve becomes more narrow, but never disappears. In a few cases, after vagal fatigue the trough did not reach the basal line completely, due to the fact that a vagal volley arriving at the pacemaker at just about the moment of a normal excitation has a small inhibiting effect upon the following beat, whereas before fatigue such a stimulation had no effect on that beat.

Experiments on turtles gave results indicating the same dependence of the vagal effect on the cycle phase. In the experiment of figure 1, which was recorded at about $\frac{1}{6}$ of the normal recording speed, it is easily seen that the second stimulation, although it occurs only approximately $\frac{1}{10}$ of a cycle phase later, has a quite different effect upon the pacemaker as expressed by the movements of the sinus venosus, which are clearly seen in the tracing of the right auricle. Measuring out curves taken at a higher speed, the same double mechanism of quick and slow A.C. liberation was found in all experiments (fig. 5). This dependence on the cycle phase was never completely absent, but was not always so great as in the eel experiments.

Is the dependence on the pacemaker activity a specific feature of the chronotropic vagal effect or present also in other vagal actions? The distribution of

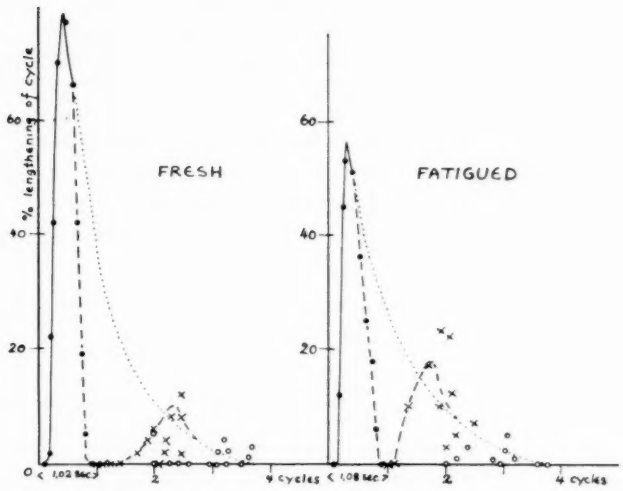

Fig. 4. Eel. A.C. curve before and after vagal fatigue. Both vagi stimulated simultaneously. 6/28/35.

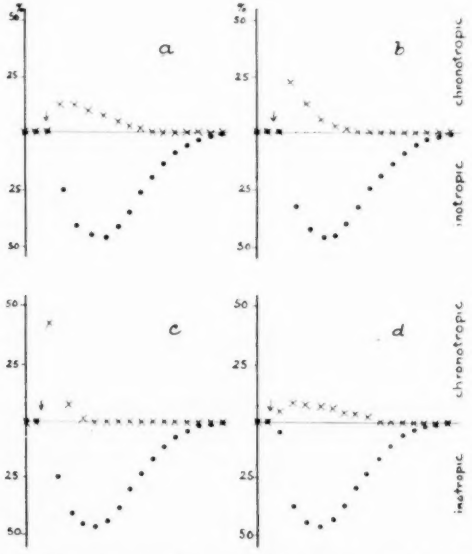

Fig. 5. Turtle. Chronotropic and inotropic (right auricle) effects. Time between arrival of vagal volley and next expected beat in a = $\frac{1}{5}$, in b = $\frac{1}{2}$, in c = $\frac{3}{5}$ and in d = $\frac{4}{5}$ of a normal cycle length. Right vagus, condenser discharges of equal strength. 12/10/35.

the action of the right and the left vagus upon the different parts of the eel and turtle heart I found, generally speaking, in accordance with previous work of others (Gaskell, 1883; Garrey, 1911, 1912; Gilson, 1932, 1933; Lee, 1935; Fredericq, 1936). In those turtle experiments, in which the movements of the different parts of the heart were recorded simultaneously, it could be demonstrated that, even when the chronotropic effect was markedly dependent on the cycle phase, the inotropic effect upon the right auricle was completely independent of the cycle phase (fig. 5). Even when the vagal volley reaches the pacemaker at such a

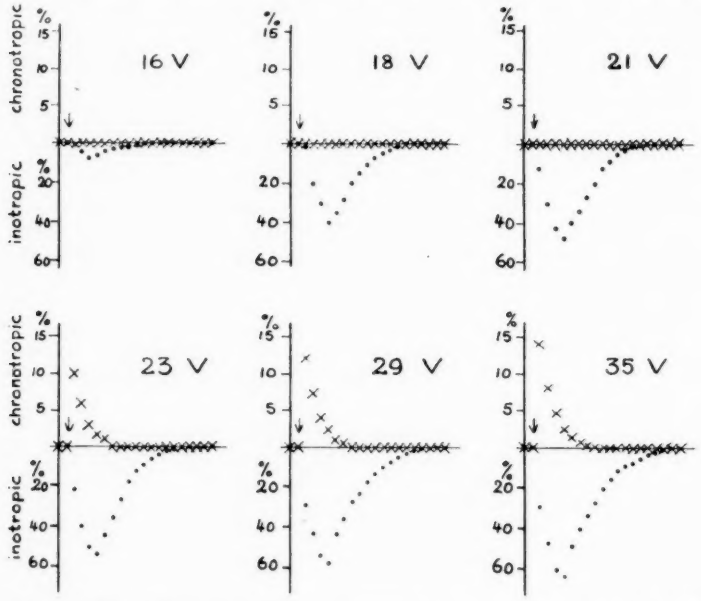

Fig. 6. Turtle. Chronotropic and inotropic (right auricle) effects. Stimulations always 1/5 cycle length after a normal beat. Discharges of a 1μF condenser, voltage increased. Right vagus only. 12/3/35.

time that the chronotropic effect is much delayed, its maximum action is reached distinctly earlier than the maximum of the auricular inotropic effect, the time relations of which correspond completely to those reported by Gilson (1932).

In five out of 27 turtles it was possible to demonstrate clearly by a threshold method that in the right vagus different fibres convey the impulses for chronotropic and inotropic action (fig. 6). In these experiments there was not the least indication that the intensity of the chronotropic effect has any influence upon the inotropic effect in the right auricle.

In not a single one of my experiments, either with the eel or the turtle, were single vagal volleys able to block completely the conduction from the auricle to the ventricle, even using both vagi simultaneously. The lengthening of conduction time between right auricle and ventricle, as found by measuring the electrocardiograms, occurs more or less parallel to the inotropic effect upon the right auricle and was independent of the cycle phase.

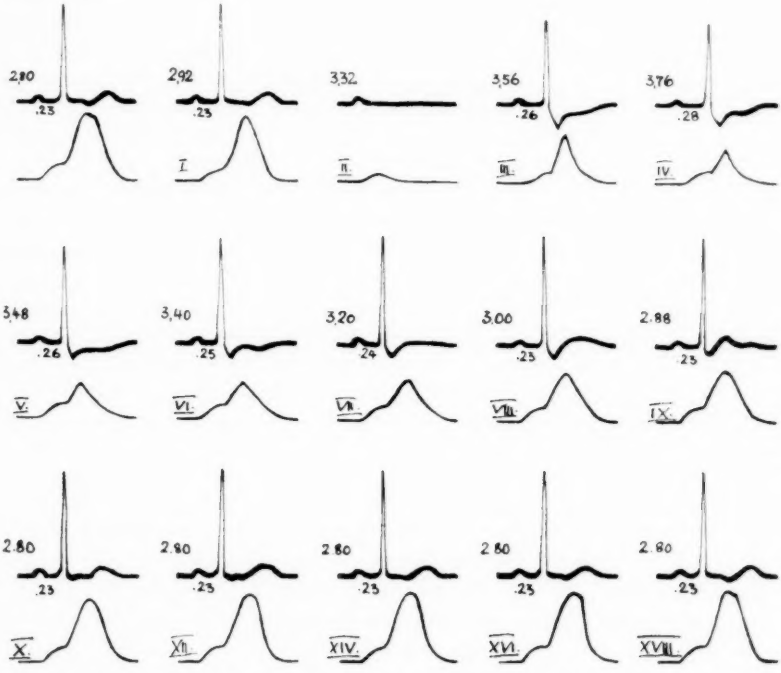

Fig. 7. Turtle, ventricle cut away completely; one electrode on right vena cava, other on tip of right auricle. Lower curve mechanogram of right auricle with cava movements recognizable. Numbers above electrogram: distance of pacemaker wave from preceding pacemaker wave in seconds. Numbers under electrogram: pacemaker-auricle conduction time. Roman numbers: number of beats after stimulation of right vagus. 12/10/35.

The action of the pacemaker was just detectable in a few of the electrograms obtained from turtles, from which the whole ventricle was removed, and in which one of the electrodes touched the right vena cava and the other the tip of the right auricle. However, when after vagal stimulation apparently no conduction to the right auricle occurred, the electric representation of the pacemaker was not altered (fig. 7). The graphic records

of the movements of the sinus venosus did not disappear completely. Besides this a complete blocking of the conduction occurred most irregularly, often being present after a stimulation of certain strength at a certain cycle phase, then missing again. But when this blocking was present, it was obvious that this dromotropic (pacemaker to auricle) effect occurred later than the maximal chronotropic effect, even when the latter was maximally delayed by the choice of the cycle phase at which the stimulus was applied (fig. 7).

The action of eserine, atropine, and acetylcholine. Eserine injections (0.1 to 0.2 mgm.) into the vena jugularis externa of the turtles increased and prolonged to a marked extent the inotropic effect of vagal stimulation upon the auricle. Its chronotropic effect is only slightly altered, but its dromotropic effect upon the conduction from the pacemaker to the auricles is increased.

Small doses of atropine sulfate (0.01–0.1 mgm.) had generally the same depressing or abolishing effect upon the chronotropic and inotropic vagal actions. When only the inotropic action was depressed, the dependence of the chronotropic action on the cycle phase was still present. But as in the experiment with vagal fatigue, the quick A.C. liberating process was more depressed than the slow one. Smaller doses of atropine had no effect at all, and I could never observe an increased excitability of the vagal mechanism as described by Gremmels (1935) for cats and rabbits after very small doses of atropine.

Injection of acetylcholine (0.0003–0.001 mgm.) exerted a marked inotropic action upon the auricle, but had only little influence upon the pacemaker and upon the size of the movements of the right vena cava although the conduction to the auricles might be blocked completely.

Discussion. *The double mechanism of A.C. liberation for the chronotropic vagal action,* as found in my eel and turtle experiments, seems to represent a general principle. Brown and Eccles (1934), whose paper I saw only after my eel experiments had been finished, report that they did not always obtain smooth A.C. curves in their cat experiments. In a relatively large number of cats they found A.C. curves of a double wave type, as represented in the lower inserted curve of figure 4. Brown and Eccles measured the time in seconds, instead of in normal cycles as I did, and since they used as zero time the moment of stimulation instead of the arrival of the volley, they were probably not aware of the fact that the deepest point of the trough, which produces the double wave curve, is always just about one cycle length later than the arrival of the volley. Fortunately they have stated for each series the cycle length, which I have indicated in their curves copied in figure 4. Furthermore, it is obvious from their data that the single wave type curve always occurs in animals if the normal cycle is short, but the double wave type when the

normal cycle is long. This explains, too, why they were able in some cases to change the double wave curve into a single wave curve by previously started and maintained weak stimulation of the accelerator nerves.

In experiments of Brown and Eccles the double wave curve represents the same phenomenon as in my experiments, as is shown by the further fact that in the case of A.C. curves of the single wave type, all their experimental points show almost no deviation from the drawn curve, while in the double wave type A.C. curves, only the first part of the drawn curve is readily determined by the experimental points.

Brown and Eccles were not able to offer a good explanation for the trough in their curves. They exclude with good reasons 1, a transient change of the pacemaker to another center; 2, a transient acceleration introduced within a single inhibitory effect; 3, after-discharge from postganglionic neurones; 4, a secondary A.C. accumulation due to diffusion from surrounding tissues. All their reasons mentioned to exclude these explanations are valid also for the experiments reported in this paper. I can only add that I could not find any evidence in the eel that any heart accelerating nerve fibres or mechanisms exist at all, which was to be expected according to the report of McWilliam (1883). In some of the turtle experiments a distinct accelerating effect was occasionally present, when using supermaximal stimulation. In those rare cases, however, the accelerating action preceded clearly the vagal action, as the first one or two cycles were shortened and the strength of the auricular beats increased, while the next cycles showed distinct lengthening and the beats were diminished in size.

The final explanation of Brown and Eccles for the trough in their A.C. curves is rather unsatisfactory. They refer to experiments by Eccles and Hoff (1934) with very early subsequent beats, which according to them are a comparable phenomenon. Therefore, they conclude that the pacemaker, like other rhythmic centers, contains "inhibitory components," and that these might be dissociated from the remainder of the center, consequently freeing it from the inhibitory action.

This explanation assumed that the trough in the A.C. curve is present for each single stimulation. It is evident, especially from the eel experiments, that the A.C. curve for a single stimulation (fig. 3) never exhibits any sign of a trough, while in a graph summarizing the results of a whole series of stimulations a trough reaching the base-line results (fig. 4). This is due to the fact, as pointed out already, that the curves for a single stimulation are not identical, so that when plotted together a trough must originate just about a cycle length later than the moment of the arrival of the vagal volley. All beats which could be delayed at that time are beats which have been influenced by a vagal volley preceding them by approximately one cycle length. The individual A.C. curves for these stimulations start only after a very long latent period and with only a gentle

slope. In consequence, the beats occurring at the time of the trough are not altered at all, or only very little.

The question remains: why do the latent period, velocity and duration of A.C. liberation depend on the cycle phase? One may suggest that the mechanism of A.C. liberation and the rhythmic mechanism of the pacemaker are linked closely together, using partly the same anatomical substrate. Thus, when a vagal volley arrives just at the moment when the rhythmic mechanism is about to free an excitation, the A.C. liberating mechanism is depressed, responding with a longer latent period and a slower velocity. However, the fact that about the same amount of A.C. is set free nevertheless is easily explained by the generally accepted assumption that preformed A.C. is released and that A.C. is not produced in consequence of a vagal stimulation.

The nature of the A.C. Recently several papers have been published (Feldberg and Vartiainen, 1935; Vogt, 1936; Brown and Feldberg, 1936; Feldberg and Guimarãis, 1936) presenting evidence that probably acetylcholine is not the only parasympathetic transmitter, but that the liberation of potassium ions plays an important part in the transmission of the excitation from the preganglionic to the postganglionic fibres. These authors assume that it is the potassium ions which are freed first, and which then produce the acetylcholine liberation. Howell (1906), Howell and Duke (1908), emphasized thirty years ago that in the heart K is freed in consequence of vagal stimulation and that these K ions produce the inhibition. Lehnartz (1936), repeating Howell's experiments, came to the conclusion that in the heart the output of K is associated with the inhibitory process but is not the true transmitter of the vagus impulses. He still regards acetylcholine as the transmitter. Armstrong (1936) found that acetylcoholine has a chronotropic effect on the heart of Fundulus embryos only after the growing nerves had reached the heart. Therefore, he assumed A.C. to be liberated only on the intramural synapses.

All of this evidence suggests a discussion of the quick and slow A.C. liberating mechanism from the point of view that the freed substances in both processes are not identical. One may assume that the quick liberating mechanism is producing acetylcholine, while the slow process liberates K ions. The fact that atropine depresses the quick mechanism more markedly than the slow one would support this assumption. Vagal fatigue depresses the quick and increases the slow process. Furthermore, eserine increases and prolongs the inotropic vagal effect much more markedly than the chronotropic effect. That the substance freed during chronotropic vagal actions is not pure acetylcholine is demonstrated by the weak action of injected acetylcholine upon the pacemaker in my own experiments, in those of Cope and Coombs (1935) in cats, and in the frog experiments of Bacq (1935). The A.C. liberated during marked vagal

inhibition of the pacemaker must reach the right auricle. Nevertheless, no influence on the size of the chronotropic effect upon the auricular inotropic effect is present (figs. 5 and 6). According to the experiments of Fredericq (1935) with quite a different method, the A.C. liberated at the sinus has only a weak influence upon other parts of the heart. Although all this evidence points out clearly that we are probably dealing with different substances in the vagal mechanism, the experimental results are still too contradictory to allow the postulation of any theory about the particular rôle these different substances play.

SUMMARY

1. In the eel the chronotropic effect of a single vagal volley is markedly dependent on the cycle phase at which the volley reaches the pacemaker. In turtles the same dependence of the vagal effect on the cycle phase is detectable.

2. It is assumed that around the pacemaker there are two mechanisms of liberation of an acetylcholine-like substance (A.C.): a quick mechanism with a short latent period, and a slow mechanism with a long latent period. The degree to which each of these liberating processes comes into play depends on the cycle phase. Vagal fatigue decreases the importance of the quick, and increases the importance of the slow A.C. liberating mechanism.

3. Neither the dromotropic nor the inotropic vagal action is dependent on the cycle phase.

4. Eserine prolongs markedly only the inotropic and dromotropic vagal effect. Atropine depresses the chronotropic effect as well as the inotropic effect. Small doses of atropine, however, depress the quick chronotropic A.C. liberation more than the slow one. Acetylcholine acts more strongly on the inotropic and dromotropic mechanism than on the chronotropic.

5. The experimental results are interpreted as indicating that the different vagal fibres affecting the heart are not only functionally distinct, but that there exist several chemical transmitters for the vagal action.

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THE EXTINCTION OF STARTLE RESPONSES AND SPINAL REFLEXES IN THE WHITE RAT[1]

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A conditioned response ultimately disappears if the conditioned stimulus is repeated without reinforcement. This decline of the response during repeated stimulation was called experimental extinction by Pavlov (1927). The disappearance of unconditioned responses upon repeated stimulation has been observed in the laboratory by Rademaker (1931) and is common in daily life. The nearest approach to this phenomenon in spinal reflexes is "fatigue" or failure of a reflex response during tetanic stimulation (Sherrington, 1906, p. 218; Forbes, 1912). The present investigation is a study of the general phenomenon of extinction of reflex responses in terms of known physiological mechanisms.

We have conducted experiments on a startle leg response to auditory stimulation and on several spinal reflexes in response to mechanical and electrical stimulation. Electrical, observational and kymographic methods of recording were utilized, the latter with spinal animals only. White rats (4–6 months old), highly inbred descendants of Wistar stock, were used.

The startle response. In studying the startle response, a rat was placed in a zipper bag similar to that of Shadle and Skarupinski (1935), but with holes cut so that the rat's hind legs and tail protruded and, if required, a portion of the rat's back could be exposed. The animal was then loosely tied on an adjustable stand below which its legs appeared. In this position it usually remained quiet for several hours. Concentric needle electrodes were inserted into various leg muscles, usually the gastrocnemius. The animal and stand were placed inside a relatively soundproof box, and approximately one hour was allowed for adaptation before beginning the experiment. The box also contained a telegraph-key sounder and a small 1.5 volt lamp which was usually kept lighted throughout an experiment. The animal could be observed through a small glass window. Wires led outside the box to a battery and switch which actuated the sounder and to an amplifier. Muscle potentials were recorded with a Matthews oscillo-

[1] A portion of the cost of this investigation was met by a grant to W. S. Hunter from the American Academy of Arts and Sciences.

graph and loud speaker. Stimulation by the telegraph sounder consisted of a brief click which, if above threshold value, elicited a leg response. (The threshold for this response was usually higher than that for the pinna reflex.) No quantitative measurements of the sound were made, but variations in intensity were produced by altering the tension on the sounder. When repetitive stimulation was used the sounder was activaetd through contacts controlled by a telechron clock, otherwise stimulations were controlled by a hand switch.

An electrical response in the gastrocnemius muscle is shown in figure 1. The startle response consisted of a brief burst of discharges in the group of muscle fibers from which the potentials were recorded. Frequently each unit fired only once, but sometimes there was an after-discharge in which each unit fired rhythmically. The latency varied over a wide range for the different units in the response, but the latency of the fastest unit was usually between 15 and 25 ms.

The effect of repeating the click stimulus once every 15 seconds is shown in figures 1 and 2B. For a number of stimulations (30 in the experiment of fig. 2B) there was a response as judged by observation through the window of the box and by the disturbance in the loud-speaker. Then the response became reduced, irregular for 35 more (see 2B), and finally disappeared. Photographic records made from time to time during this extension of the response are shown in figure 1. During the course of the extinction the response diminished in size as shown by a diminution in the duration of the after-discharge and a decrease in the number of active motor units until ultimately no units responded. As units dropped out during the extinction, their latencies did not alter appreciably and their magnitudes did not change. The effect is as if the threshold for different units became higher, each dropping out at some level without undergoing any change in its time relations.

As shown in figure 2B, during the period when the response is irregular there appear to be waves or cycles of excitability. These are shown by intervals when the response is present followed by corresponding intervals when no responses occur. The number of responses between periods of no response is reduced; i.e., the periods when no response occurs are not lengthened. Stimulation at a faster rate, once every two or five seconds, induced extinction in a much shorter time than when stimulation intervals were ten or fifteen seconds.

Once the effect is complete to the extent that approximately five consecutive stimulations elicit no responses, the extinction may persist through many minutes even though no stimuli are given. Further stimuli strengthen the extinction and the more stimuli given after extinction, the longer does the effect last. Spontaneous recovery does usually occur, however, after 15 to 20 minutes of non-stimulation.

If, after extinction is complete, general sensory stimulation occurs, such as opening the door of the box or flashing a bright light in front of the rat, the response in most cases returns immediately to the next auditory stimulus and requires numerous repetitions before it is again extinguished.

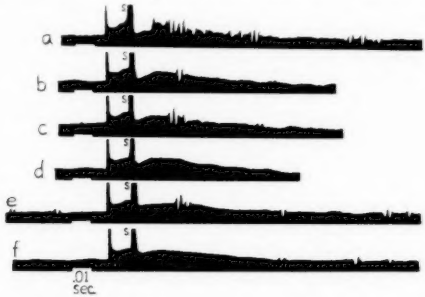

Fig. 1. Extinction of startle response in intact rat. *S*—stimulus signal. a, b, first two responses; c, 11th response; d, extinction—15th stimulus. e, Next successive response. Disinhibition by light occurred between d and e. Same units present as before extinction. f, 9th stimulus after disinhibition (extinction).

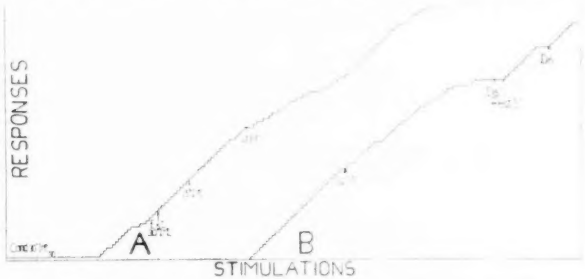

Fig. 2. "Conditioning" and extinction of startle response in intact rat. Ordinates—responses; abscissae—stimulations. Responses by observation of animal and by listening to loud-speaker disturbance.

A. "Conditioning" with sound followed by shock to foot, paired stimuli every 30 seconds. Ninety stimuli omitted from figure at *90*. *Ext. 30" Int.*—extinction of the conditioned response began with stimuli unreinforced, 30 second intervals. *15" Int.*—unreinforced stimuli at 15 seconds intervals. *10" Int.*—unreinforced stimuli at 10 seconds intervals.

B. Extinction of unconditioned startle response. *Sp. M.*—spontaneous movement. *Dis.*—attempted disinhibition by light. Rest 5 minutes, followed by spontaneous recovery. Second disinhibition effective because extinction not so complete as at time of first attempt at disinhibition.

This effect is shown in figures 1e and 2B. It is similar to the phenomenon called "disinhibition" by Pavlov. If the extinction proceeds to a considerable degree, a stronger stimulus is required for disinhibition than after the first few failures to respond. When the response returns, either sponta-

neously or as a result of disinhibition, the general characteristics as measured by the muscle potentials are the same as before extinction (fig. 1e). The latencies for individual units appear to be unaltered throughout the process of extinction and recovery.

In some experiments, instead of starting with a sound of an intensity which elicited a startle leg response, an intensity sub-threshold for this response was used. At each presentation the click was followed after 0.5 second by a reinforcing tetanic shock applied to the hind foot of the leg with the electrodes. In these experiments, after 75 to 100 paired presentations, a response to the sound usually appeared. The course of the establishment of the "conditioned" response is indicated in figure 2A. The nature

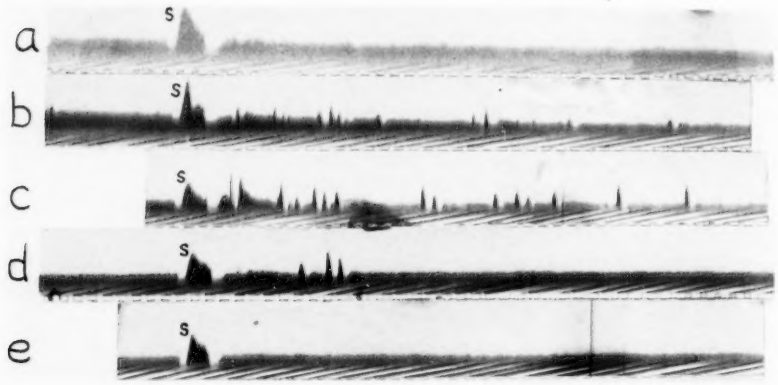

Fig. 3. "Conditioning" and extinction of startle response in intact rat. *S* = stimulus signal. Time intervals 0.01 second. a, Near beginning of conditioning, after 75 reinforced stimulation; b, during conditioning, after 20 more reinforced stimulations; c, well "conditioned" response, after 154 more reinforced stimulations; d, partially extinguished response (50 unreinforced stimuli); e, extinction complete.

of the response is shown in figure 3. The response is essentially the same as the previously described unconditioned startle response in latency, number of active units, duration of after-discharge, and frequency of individual units. Once the response was established it could be extinguished by omission of the reinforcement. The course of this extinction is similar to the extinction of the unconditioned response. In some experiments it was impossible to condition the response in approximately 150 trials. This is in keeping with the findings of many investigators who observe extreme variability among animals in their susceptibility to conditioning.

In another set of experiments the conditioning method was applied to the unconditioned startle response, using as the "conditioned" stimulus an

intensity of auditory stimulus just sufficient to evoke the response. The sound was followed 0.5 second later by a reinforcing shock. In most animals the response became irregular for a number of presentations, then it usually became stronger. The strengthening of the response by this method consisted in an increase in the number of active units and a greater duration of the after-discharge. There was no significant change in the latency of individual units.

It is questionable, in view of the nature of the response, whether any of the above experiments deal with true conditioning. It appears rather that the general excitation by the electric shock raises the excitability of the centers involved so that a sub-threshold stimulus becomes and then remains effective. The constancy of the several motor unit latencies which we have found contrasts *a*, with the decreasing latency of the gross conditioned response as found by Anrep (1920) and Hilgard and Marquis (1935); and *b*, with the increasing latency described by Switzer (1934).

The latencies of the fastest units of the startle response (15–25 ms.) are such that the response, although made by an intact animal, probably does not involve the cerebral cortex which itself shows a minimum latency of 8 ms. to auditory stimulation (Davis, 1934). Lorente de Nó (1933) has described a tensor tympani reflex to sound mediated via the inferior colliculus and superior olivary body with a total latency of 14 to 16 ms. It seems likely that the startle response involves the following path: cochlea, eighth nerve, cochlear nucleus, inferior colliculus (latency to here 2.5–3.5 ms. as determined by Kemp and Coppée (1936)) reticular nucleus in mid-brain, reticulo-spinal tract, anterior horn cells, and motor nerves to leg. Assuming 3 to 4 ms. delay in the anterior horn cells, 2 to 3 ms. for conduction on the efferent side, and 3 to 4 for end-plate delay, we are left with 4.5 to 7 ms. from the colliculus to the anterior horn cells, a path which probably involves two synapses. Previous investigators (Culler and Mettler, 1934) report the conditioning of decorticate animals, and auditory startle responses have been observed in cats in which the brain was transected through the anterior colliculi (Forbes and Sherrington, 1914).

Spinal reflexes. The preceding experiments demonstrate, we believe, extinction, reinforcement and disinhibition of an essentially collicular response. It is important, therefore, to ascertain whether these are properties of all forms of behavior (and of all nerve centers), and we have thus extended the work to include experiments on several spinal reflexes.

Chronic spinal preparations were made either by inserting a needle between the vertebrae and so transecting the cord, or, more often, by exposing the cord by the removal of two mid-thoracic vertebral dorsal arches and then transecting with a knife. Most of the animals were kept for one or two weeks, and there was a complete failure of cephalad and caudad conduction across the transection throughout the period. The following

reflexes were studied: 1, leg response to tap on the rump and to tap on the tail; 2, flexion of base of tail to tap near tip of tail; 3, tail flexion and leg response to tetanic stimulation of the tail; and 4, crossed leg response to direct electrical stimulation of the contralateral saphenous nerve. Of these reflexes the least satisfactory was the response to the tap on the rump. The tap stimulus was administered with the head of a tack held in a lever

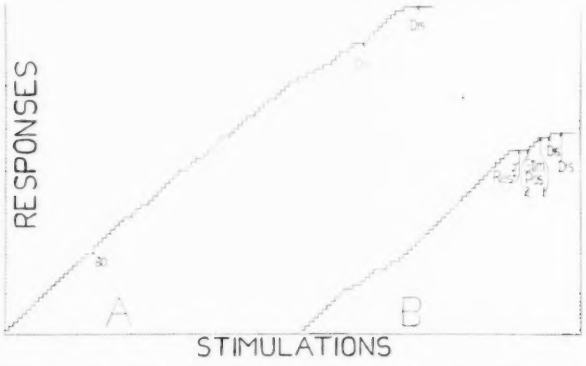

Fig. 4. Extinction of tail response in spinal rat. *A*, extinction to electric shock on tail. Eighty stimuli and responses omitted as shown. *Dis*—disinhibition by pinch; second ineffective because extinction more complete than at first. *B*, extinction to tap on tail. Rest two minutes—no recovery. *Stim. Pos. 2*—tap shifted approximately 2 mm. followed by response. *Stim. Pos. 1*—tap returned to first position, response still extinguished. *Dis.*—disinhibition by pinch.

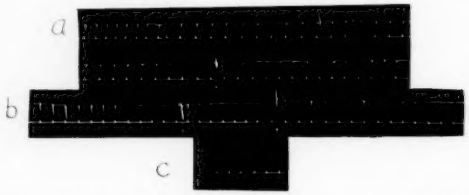

Fig. 5. Extinction of crossed leg response to electrical stimulation of saphenous nerve, spinal rat. A: stimulus near threshold, gradual diminution and ultimate extinction of response, pinch on foot at arrow caused disinhibition. B: Continuous with A. Two disinhibitions at arrows, subsequent extinction. C: Effect of same stimulus after crushing nerve centrally.

which was activated by the armature of an electromagnet. In all cases the tail was held immobile by adhesive tape at the area stimulated. Electric recording was by concentric needle electrodes placed either in the ventral base of the tail or in the gastrocnemius of one hind leg.

The records of figures 4 and 5 are typical and show that all of these responses eventually become extinguished during repetitive stimulation.

The kymograph records clearly show that as stimulation continues the response diminishes in magnitude. When the stimulus was near threshold, the spinal responses diminished and finally disappeared after fewer stimulations than when the stimulus was stronger. In fact with very strong taps and electrical stimulations, the response was frequently not extinguished, although it was reduced in size. When a response was extinguished to a given stimulus intensity, an increase in the intensity would again arouse the response.

The electrical measurements show that as the spinal responses become extinguished the number of active units and the duration of the after-discharge are reduced with no diminution or increase in the latency for a given unit. As with the startle response, the latency of the fastest unit means little because during extinction this unit may be extinguished before one which is a little slower, and after extinction of the first unit this slow one becomes the leading unit of the response.

In studying extinction with direct nerve stimulation, a spinal rat was etherized and the saphenous nerve of one leg was exposed and freed from the surrounding tissue. Insulated silver wire stimulating electrodes were placed under the nerve. The incision was closed and the rat was allowed to recover, then placed on its back in the zipper bag, tied on the platform support with its legs raised. Tetanizing shocks, 0.5 second duration, of near threshold value for the contralateral leg response, were delivered at the rate of 4 per minute by the telechron timer. The kymograph records of figure 5 are typical. Prior to the beginning of record A, the rat had received 83 shocks with no extinction. The shock was decreased, and the record shows 32 more responses followed by 3 shocks without a response. Pinching the tail, at the point marked with an arrow in the figure, disinhibited the response. Later in the experiment, the response was twice again extinguished and disinhibited (record B). In order to show that the response to the shock was neurally controlled rather than due to electrical spread or to mere mechanical jarring from the operated leg, the nerve was now pinched centrally from the electrode. Record C shows that the shock no longer produced a response in the leg.

Figures 4A and 4B also show disinhibition of spinal reflexes as a result of strong afferent stimulation resulting from pinching the tail or the foot of the rat. We have, in addition, secured records of apparent self-disinhibition. Here after the response has been extinguished and the rat has, for some reason, struggled "spontaneously," the reflex reappears to the subsequent stimuli. Such self-disinhibition may account for much of the variability found in extinction records.

INTERPRETATIVE COMMENTS. The extinction here described is considered as a central phenomenon. Evidence is unanimous that at frequencies of stimulation such as one every 10 or 15 seconds no sensory adaptation

occurs. This is certainly true for the auditory tracts (Davis et al.) and for muscle and tactile receptors. Further, the fact that we find extinction of spinal reflexes elicited by direct stimulation of the nerve shows that sensory adaptation is not involved. Similarly, there is no evidence that the motoneurones and muscles themselves show any such decline in responsiveness.

The experiments illustrated in figure 4B show that the extinction effect must occur antecedent to the final motoneurones. Here a tail response to a tap on the tail was extinguished. Then the tapper was moved 1 to 2 mm. to activate other receptors and a response of the original motor units as well as of additional motor units occurred at once to the tap in the new location. This is in harmony with the evidence recently presented by Loucks (1935) indicating that conditioning must occur antecedent to the pyramidal cells of the cortex. If this argument holds, a reflex in which on internuncial neurone occurs should not be capable of extinction. We have obtained a liminal knee jerk in a spinal cat 980 successive times in one experiment and 800 times in another with no diminution in the response (stimulation frequency 4–6 per min.). In the same animal a reflex in which internuncial neurones are involved was extinguished in 27 trials. The evidence as a whole indicates that extinction and disinhibition occur beyond the sensory neurone and ahead of the final motoneurone, presumably at some of the internuncial synapses.

What is the nature of the change in conduction that occurs during extinction? It cannot be inhibition in the Sherringtonian sense since this central inhibition lasts only 100 to 300 ms. (Creed, Denny-Brown, et al., 1932, Ch. 6). For the same reason it cannot be equilibration as described by Gerard and Forbes (1928). It is unlikely that it is fatigue in the usual sense of the accumulation of waste products and the exhaustion of reserves. The circulation is entirely adequate to prevent such effects when stimuli fall so far apart as in the present experiments. Furthermore, the effect occurs more quickly at low (near-threshold) than at high intensities of stimulation, although the stronger stimuli should produce the greater fatigue and exhaustion. In addition, the fact that strong general excitation can wipe out (disinhibit) the effect is incompatible with notions of fatigue.

Dusser de Barenne and McCulloch (1934) found an "extinction" of responses to stimulation of the motor cortex when periods of stimulation fell at intervals of approximately 13 seconds. Fatigue, or pseudo-fatigue of spinal reflexes, developed with a much higher frequency of stimulation than we have used, is a central phenomenon (Sherrington, 1906; Forbes, 1912; Lee and Everingham, 1909). Whether such "reflex fatigue" and Pavlovian "inhibition" are the same is at present a verbal matter to be settled, if at all, in terms of experimental methods employed.

The extinction process involves the gradual dropping out of active units and the diminution in the duration of their activity. The effect may persist for many minutes, and it can be eliminated (disinhibited) by general excitation. These facts indicate that extinction is a gradual lowering of the excitability; the excitability may remain low for a relatively long period, or it may be raised by disinhibiting stimuli. Conversely, *increased* excitability of long duration can be produced by reinforcement of a weak stimulus by general excitation (electric shock).

SUMMARY

A reflex startle leg response in the rat, probably mediated by the colliculus, occurs to brief auditory stimulus. When the stimulus (a click) is repeated at intervals of 10 to 15 seconds the response weakens and ultimately disappears. This extinction consists of a gradual diminution in the number of active motor units, a decrease in the duration of the after-discharge, but no change in latency for any units.

After complete extinction the response spontaneously recovers, often after 5 to 30 minutes' rest without stimulation. Recovery may occur at once if the animal becomes generally excited (disinhibition).

Reinforcement of a sub-threshold sound by a shock to the foot raises the excitability of the center so that the response appears after many paired stimulations. This "conditioned" response can be extinguished by the same method as the unreinforced response.

The following spinal reflexes can be extinguished with stimuli repeated at intervals of 10 to 15 seconds: leg and tail response to tap on tail, to tap on back, to electric shock on tail, crossed leg reflex to stimulation of saphenous nerve. After extinction the responses can be brought back (disinhibited) by strong general excitation (e.g., pinch on foot).

These effects are interpreted as being slow, semi-permanent shifts in excitability of some part of the reflex arc between the sensory neurones and the final motoneurones.

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SPINAL VASOMOTOR REFLEXES ASSOCIATED WITH VARIATIONS IN BLOOD PRESSURE[1]

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It is well known that the aortic arch and the carotid sinuses constitute vascular zones sensitive to blood pressure, and are the seat of reflex regulation of cardiac frequency and vascular tone. In a publication in 1929 (1), one of us pointed to the existence of other reflex vascular zones, likewise sensitive to alterations in blood pressure, and located probably in the vascular area bounded by the arch of the aorta and the bifurcation of the iliac arteries. The present studies are concerned with the localization and rôle of these latter pressure-sensitive vascular zones.

METHOD. All experiments performed in the first part of these studies were carried out with the same basic technique. Dogs, anesthetized with chloralosane, were prepared in the following manner: the spleen of a dog B was perfused by a dog A; by making anastomoses with the aid of Payr cannulae, the splenic artery of dog B was joined to the carotid artery of dog A, and the splenic vein of dog B was joined to the external jugular vein of dog A. The extrinsic innervation of the spleen of dog B remained intact. The vascular tone of the perfused spleen of dog B was registered by means of a plethysmograph. The carotid sinus nerves and the vagus-depressor (aortic) nerves of dog B were cut to exclude the reflex action of the pressure-sensitive vascular zones in the carotid sinuses and the aortic arch. The blood pressure of dog B was modified, either by hemorrhage or the intravenous injection of serum, blood or substances which cause hypertension (adrenaline, ephedrine).

In a first series of experiments, it was found, in confirmation of the observations previously made by one of us (1), that in the absence of the pressure-sensitive reflex zones in the aortic arch and in the carotid sinuses, modifications in the general blood pressure still cause inverse and com-

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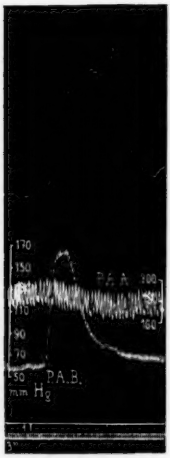

Fig. 1. Dog A, 17.0 kgm.; dog B, 19.0 kgm. Chloralosane anesthesia. The spleen of spinal dog B is perfused by dog A and is connected with its owner (dog B) only by means of the extrinsic splenic nerves. From above downward: volume of the spleen of dog B; general blood pressure of dog A (P.A.A.); general blood pressure of dog B (P.A.B.). At *1*, intravenous injection of 0.1 mgm. adrenaline into dog B. Note dilatation of the spleen of dog B in response to hypertension in spinal dog B.

Time in intervals of 3 seconds.

pensatory reactions on the vascular tone. This was demonstrated by the occurrence of dilatation of the perfused spleen of dog B in response to arterial hypertension in dog B, and constriction of the perfused spleen of dog B in response to arterial hypotension in dog B. Experiments previously carried out in this laboratory (1, 2, 3), had demonstrated that neither adrenaline nor modifications in the general blood pressure influences the tone of the vasomotor centers in a direct manner. Other possibilities were therefore investigated.

1. Another group of experiments was undertaken to determine whether the vasomotor reactions described above, in the dog deprived of the vascular zones in the aortic arch and carotid sinuses, persist in a dog deprived of his encephalo-bulbar centers.

METHOD. The technique described above was employed, with the following additions: the neck of dog B was severed at the level of the second or third cervical vertebrae by means of a special crusher (1); the trunk of dog B was kept alive by artificial respiration.

RESULTS. When variations in the arterial pressure of trunk of spinal dog B were brought about, there occurred vasodilatation of the perfused spleen of dog B when the arterial pressure in the trunk of dog B was raised by adrenaline (fig. 1), ephedrine, or pituitrin, and a vasoconstriction of the perfused spleen of dog B following a lowering of the blood pressure in the trunk of dog B.

The vasomotor tone of the spleen is therefore regulated in a neurogenic manner by alterations in the general blood pressure in an animal deprived not only of its four buffer nerves but also of its encephalo-bulbar vasomotor centers. Certain questions concerning this dependence of the vascular tone of the spleen on the general blood pressure in a spinal dog could be answered by these experiments. The possibility of a specific action of adrenaline on the spinal vasomotor centers was at once excluded, since the same effect on the spleen was obtained when other substances were used to raise the blood pressure. The possibility that the reactions in the spleen might be caused by a "washing-out" of the spinal vasomotor centers due to a sudden increase in

the spinal cord circulation in an animal with abnormally low blood pressure could be excluded by the observation that vasodilatation of the perfused spleen occurred also in experiments where the initial blood pressure

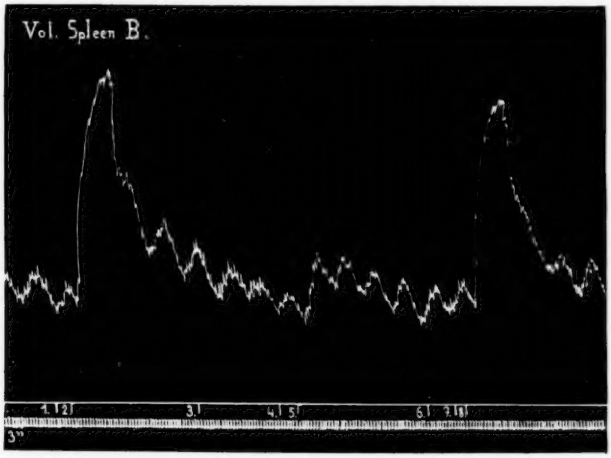

Fig. 2. Dog A, 19.0 kgm.; dog B, 22.0 kgm. Chloralosane anesthesia. The carotid sinus nerves, both vagi and the aortic (depressor) nerves of dog B have been cut. The spleen of dog B, perfused by dog A, is connected with its owner (dog B) only by means of the extrinsic splenic nerves.

The numerals refer to the following procedures carried out on dog B. At:

1. Occlusion of the aorta just *below* the origins of the celiac and superior mesenteric arteries.

2. Injection into the external jugular vein of 0.2 mgm. adrenaline. Note marked reflex vasodilatation of the spleen in response to arterial hypertension.

3. Occlusion of the aorta ended.

4. Occlusion of the aorta *above* the origins of the celiac and superior mesenteric arteries.

5. Injection into the external jugular vein of 0.2 mgm. adrenaline. Note very slight dilatation of the spleen in response to arterial hypertension.

6. Occlusion of the aorta ended.

7. Same as *1*. Occlusion of the aorta just below the origins of the celiac and superior mesenteric arteries.

8. Same as *2*. Injection into the external jugular vein of 0.2 mgm. adrenaline. Note marked reflex dilatation of the spleen in response to arterial hypertension.

Time in intervals of 3 seconds.

was normal, and even above normal, before hypertension was artificially induced.

2. Since a direct action on the spinal vasomotor centers could be ruled out, a second hypothesis seemed permissible: could there be a reflex

mechanism comparable to that which regulates vasomotor reflexes originating in the aortic arch and the carotid sinuses?

To answer this question, in a first series of experiments, variations in the arterial pressure of a dog B were effected alternately during occlusion of the aorta above and below the origins of the celiac and the superior mesenteric arteries. Under these conditions, it was found (fig. 2) that, when the blood pressure was raised in a dog B, deprived of all other vasosensitive zones, in that portion of the aorta where the celiac and superior mesenteric arteries originate, an intense vasodilatation occurred in the perfused spleen of dog B; on the contrary, when the vascular area supplied by the celiac and the superior mesenteric arteries was excluded, and then elevation of the general blood pressure brought about, only a very slight vasodilatation of the perfused spleen occurred. It was also found that acute destruction of the spinal cord of the spinal dog caused complete disappearance of the splenic vasomotor reflexes just described.

From these experiments it appears that the vasomotor reactions in the spleen, brought about by variations in the general blood pressure, are reflex in origin, and are determined, for the most part, by the effect of blood pressure on a pressure-sensitive innervation located in the organs and structures supplied by the celiac and superior mesenteric arteries.

3. The next problem to be considered was whether these spinal vasomotor reflexes caused by variations in general blood pressure, and more especially, by arterial hypertension in the spinal dog, affect not only the vasomotor tone of the spleen, but also other vascular areas. A new series of experiments was carried out to investigate this question.

Method (fig. 3). The method of Delezenne was employed. The leg of a dog B, connected with the trunk of a spinal dog B only by the sciatic, crural and obturator nerves, is perfused by a dog A. By means of Payr cannulae, anastomoses are effected between the crural artery of the leg of dog B, and the carotid artery of dog A, and between the femoral vein of dog B and the external jugular vein of dog A. The vasomotor tone of the perfused leg of B is registered by the recurrent arterial pressure in the profunda branch of the femoral artery (method of Nolf), or by the lateral arterial pressure in the artery of perfusion.

Results. When the general blood pressure in the trunk of B was raised, (fig. 4) vasodilatation of the perfused leg of B occurred. A return to normal blood pressure or a hypotension in trunk B caused a vasoconstriction of the perfused leg of dog B.

The arterial pressure of the spinal dog, therefore, influences by reflex means, not only the vascular tone of an abdominal viscus—the spleen, but also the peripheral vascular tone, as was noted in the perfused leg.

4. These spinal vasomotor reflexes, as well as the carotid sinus and aortic arch vasomotor reflexes were completely absent in the totally sym-

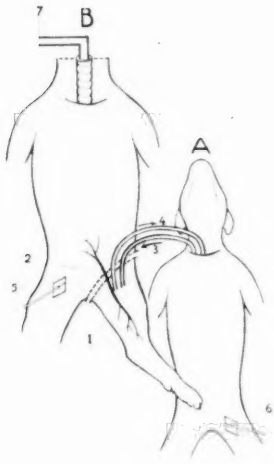

Fig. 3

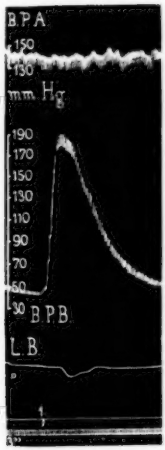

Fig. 4

Fig. 3. Diagrammatic representation of the technique employed in the perfusion by dog A of the hind leg of a spinal dog B, which is connected with its owner (dog B) only by the sciatic, crural and obturator nerves.

1. Hind leg of dog B.

2. Intact sciatic, crural and obturator nerves.

3. Arterial pathway from the carotid artery of dog A, anastomosed to the crural artery of the hind leg of dog B.

4. Venous pathway from the femoral vein of the hind leg of dog B, anastomosed to the external jugular vein of dog A.

5. Cannula in the femoral artery of the intact hind leg of dog B for the registration of the general blood pressure of dog B.

6. Cannula in the femoral artery of dog A for the registration of the general blood pressure of dog A.

7. Tracheal cannula, which is connected to an artificial respiration apparatus.

The spinal cord of dog B is severed at the level of the 2nd or 3rd cervical vertebra. The carotid sinus nerves, and both vagus and aortic (depressor) nerves have been cut.

Fig. 4. Dog A, 17.0 kgm.; dog B, 11.0 kgm. Chloralosane anesthesia. Prepared according to the technique illustrated in figure 7. The vasomotor tone of the perfused leg is measured by the lateral pressure in the hind leg of dog B. Vasodilatation—downward.

From above downward: arterial pressure of dog A (B.P.A.); blood pressure of dog B (B.P.B.); lateral pressure of the perfused hind leg of dog B (L.B.).

At *1*, intravenous injection into spinal dog of 0.4 mgm. adrenaline.

Note reflex vasodilatation in the perfused leg of dog B in response to arterial hypertension in spinal dog B.

Time in intervals of 3 seconds.

pathectomized dog, from whom the two paravertebral chains had been removed several months previously. In this experiment, the spleen of dog B (the totally sympathectomized dog) was perfused by dog A, and

was in connection with its owner (dog B) only by means of its extrinsic splenic nerves. It was also of interest in this experiment that electrical stimulation of the central end of the vagus nerve did not cause any constriction of the perfused spleen, nor any elevation of the general blood pressure.

The observation of the absence, in the totally sympathectomized dog, of the vasomotor reflexes normally induced by blood pressure changes in the carotid sinus is confirmatory of the work of Bacq, Brouha and Heymans (4) on this point. The absence of arterial hypertension on stimulation of the central end of the vagus nerve in the dog, totally sympathectomized for several months, is in accord with the similar observation of Heymans, Bouckaert and Jongbloed (5) on the acutely sympathectomized dog.

Discussion. These experiments have demonstrated that in the dog deprived of both vagi and of the vasosensitive zones in the aortic arch and carotid sinuses, increase and decrease of the general blood pressure cause, respectively, vasodilatation and vasoconstriction in the splanchnic circulation (spleen) and in the periphery (leg). These vasomotor reactions are not of direct central origin. Nor are they of reflex origin from the lungs (6), since section of both vagi does not interfere with the demonstration of the vasomotor reactions. These reactions are still present in the spinal dog, but disappear completely after the acute destruction of the cord in the spinal dog.

These vasomotor reflexes caused by variations in the blood pressure in the spinal dog are, in great part, spinal reflexes originating in vascular zones sensitive to pressure, and located in the area supplied by the celiac and superior mesenteric arteries. The origin of these spinal vasomotor reflexes might correspond to the reflex mesenteric sensitivity observed by Gammon and Bronk (7), and located by those authors in the Pacinian corpuscles, although their results are not compatible with ours. In a series of experiments, which gave results which those authors regard as suggestive rather than conclusive, Gammon and Bronk observed reflex vasoconstriction in response to distention of the mesenteric vessels, while we obtained reflex vasodilatation following an increase of pressure in the splanchnic circulation, and a reflex vasoconstriction following a decrease of pressure in this circulation.

These spinal vasomotor reflexes which adapt vascular tone to the general blood pressure, however, do not appear to play an important part in the automatic and proprioceptive regulation of the general blood pressure, as do the vasosensitive zones in the aortic arch and the carotid sinuses. In fact, occlusion of the celiac and superior mesenteric arteries, that is, hypotension in the splanchnic organs, does not cause a reflex vasomotor reaction sufficient to provoke any augmentation of the general blood pressure.

It is probable that the spinal vasomotor reflexes under discussion play a rôle in the distribution of blood in the deep abdominal circulation and in the periphery. These reflexes may conceivably have a more far-reaching influence under certain special conditions, and in a minor and accessory manner, may join with the vascular zones in the aortic arch and the carotid sinuses in the regulation of the circulation and the general blood pressure.

SUMMARY

1. In the dog deprived of the vascular zones in the aortic arch and the carotid sinuses, increase and decrease of the general blood pressure still cause, respectively, vasodilatation and vasoconstriction in the splanchnic circulation (spleen) and in the peripheral circulation (leg). These vasomotor reactions persist after section of both vagus nerves.

2. In the spinal dog, after complete section of the neck, increase and decrease of the general blood pressure cause, respectively, vasodilatation and vasoconstriction in the splanchnic circulation (spleen) and in the peripheral circulation (leg). These vasomotor reactions disappear after the acute destruction of the spinal cord in the spinal dog.

3. The vasomotor reactions caused in a spinal dog by variations in general blood pressure are vasomotor reflexes produced, for the most part, by variations in the blood pressure in the organs supplied by the celiac and superior mesenteric arteries. Other vascular areas (thoracic, peripheral ?) also possess reflex sensitivity to endovascular pressure.

4. The spinal vasomotor reflexes induced by alterations in the general blood pressure, as well as the carotid sinus and aortic vasomotor reflexes and the arterial hypertension and reflex vasoconstriction normally induced by electrical stimulation of the central end of the vagus nerve, are absent in the totally sympathectomized dog.

5. The physiological rôle of spinal vasomotor reflexes is discussed with special reference to those reflexes brought about by alterations in the blood pressure in the abdominal circulation.

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A STUDY OF "SIMPLE DISUSE ATROPHY" IN THE MONKEY[1]

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Other than in immobilization as occurs, for example, in the treatment of fractures, there are very few conditions which cause simple disuse atrophy of skeletal muscles. Immobilization experiments have been carried out by Froboese (1), Legg (2), Thompson (3) and Lippman and Selig (4), but their results are not in agreement. Differences in technique are, no doubt, responsible for many of the discrepancies. The present study was undertaken in an attempt to clarify this problem.

METHOD. Six young macacus rhesus monkeys were employed in the experiment. By applying a body and leg cast, an attempt was made to reduce the activity of the gastrocnemius-soleus muscles to a minimum, although it was realized, of course, that muscles so immobilized are still subject to "static" activity due to "stretch" and other tonic reflexes, which influences can only be abolished by nerve section.

Before applying the plaster, the extremity was first placed in a stockinette sleeve extending distally beyond the toes and proximally high up over the abdomen. The extremity was then wrapped very thoroughly with cotton batting to prevent pressure sequellae. Preliminary experiments showed that ischemic paralysis and pressure sores would be produced very easily by improper applications of the cast, and that the success of the experiment depended primarily upon the avoidance of such complications. In applying the plaster bandages, the limb was held in a position of slight flexion at the knee, in order to obtain maximum relaxation of the gastrocnemius soleus muscles and to avoid stretching. At designated periods of one, two, three, four, six, and ten weeks, respectively, the casts were removed. Using aseptic technique, the gastrocnemius-soleus muscles were dissected from their proximal and distal attachments.

Immediately following the excision, the muscle group was weighed and then bisected longitudinally. One portion was used for histologic study and the other for chemical analyses. For histologic studies, the sections

[1] This work was carried on with the assistance of a grant from the Council on Physical Therapy of the American Medical Association.

were stained by the hematoxylin and eosin, Van Gieson,– and a modified Ranson pyridine silver–methods.

In chemical analyses, water content was determined by desiccation of the muscle over sulphuric acid under reduced vapor pressure. Total nitrogen was determined by the Kjeldahl method and the protein content calculated.

In several preliminary experiments, the atrophied muscles were tested for threshold responses to faradic and galvanic stimulation and compared with the controls.

RESULTS. Within one week after immobilization there was gross evidence of a decrease in muscle bulk. This was verified by weighing, which revealed a loss of 4.9 per cent, as compared to the control side. In the

TABLE 1

Atrophy of simple disuse

	ONE WEEK		TWO WEEKS		THREE WEEKS		FOUR WEEKS		SIX WEEKS		TEN WEEKS	
	Left Control	Right Atrophy	Left Control	Right Atrophy	Left Control	Right Atrophy	Lef Control	Right Atrophy	Left Control	Right Atrophy	Left Control	Right Atrophy
Wt. specimen on removal	12.28	11.67	12 19	10.62	13 59	11 45	21 48	15.65	20 43	14 92	14 44	9 77
Weight difference	0.61		1.57		2 14		5.83		5 51		4.71	
Per cent wt. loss	4 9%		12.8%		15.7%		27.1%		29 8%		32 5%	
Wt. ½ to be desiccated	5.53	5.37	6.25	5.15	7.61	6.16	11.63	8.74	11.78	6 56	6 80	4 48
Constant wt. after prolonged desiccation	1.46	1.38	1.57	1.35	1.88	1.49	2.90	2 24	3.04	1 68	1 73	1.25
Water content in gms.	4.06	3.99	4.67	3.79	5.73	4.67	8.73	6.50	8.74	4.88	5 17	3.23
Per cent water	73.5%	74.2%	74.8%	73.6%	75.3%	75.8%	75.0%	74.3%	74.0%	74 3%	74.5%	72.1%
Total nitrogen	0.196	0.195	0.216	0.185	0.263	0.208	0.400	0.278	0.402	0 246	0.232	0 151
Protein N × 6.25	1.225	1.217	1.351	1.156	1.645	1.299	2.502	1.742	2.511	1 537	1 449	0.943
Per cent protein	22.0%	22.5%	21.6%	22.5%	21.6%	21.1%	20.9%	21.2%	21.4%	21 9%	21 3%	21.0%
Specimen number	1	2	3	4	5	6	7	8	9	10	11	12

other specimens subjected to longer periods of inactivity (2, 3, 4, 6 and 10 weeks, respectively), the atrophy was found to be increased accordingly, as indicated in table 1.

Grossly, the atrophied muscles appeared somewhat paler than those of the control side. Microscopically, the muscle bundles were definitely smaller in diameter. The individual muscle fibers were narrower in cross-section and in longitudinal section showed prominent cross-striations, the Q bands appearing denser than normal. In cross-section, the fibers appeared more homogeneous, and Cohnheim's zones were less prominent, implying a decrease in the amount of sarcoplasm. Longitudinal striations were not observed. The sarcolemmal and muscle nuclei were not obviously increased in number nor altered in size, and there was no evidence of mito-

sis. The connective tissue elements were not increased. There was no change in the intra-muscular blood vessels. The intramuscular nerves and nerve endings were intact and normal in appearance, as demonstrated by the silver stain.

Chemical studies showed that the proportions of water and protein remained essentially the same as in normal muscle. For example, in the ten-week specimens, despite a 32.5 per cent weight difference between the atrophied and the control muscles, the variation in protein content between the two was but 0.30 per cent, and the difference in water content was too slight to have any significance.

Electrical stimulation of the atrophied muscles with faradic and galvanic current gave prompt responses similar to those obtained in normal skeletal muscle.

Discussion. Formerly, the atrophy resulting from lesions of the nervous system, as well as in the arthritides in which activity is limited, has been attributed to disuse. This concept of disuse atrophy, however, is not strictly correct and needs to be clarified.

By simple atrophy of disuse is meant that wasting of muscle tissue which results solely from a curtailment of its specific function, namely, contraction, and without any accompanying disturbance of its nerve or blood supply. The atrophy resulting from disuse was found to be a very slow process. As such, it differs greatly from muscle atrophy resulting from lower motor neuron lesions. Computation of weight loss by comparing the inactive muscles with those of the opposite extremity, is subject to error. It may be pointed out that because of the limitation of activity of the one extremity, there was an increased use imposed upon the opposite one, which might result in some hypertrophy. Such a criticism seems quite justified. On the other hand, when one compares the general behavior of the animals used with that of the untreated animals, it is quite evident that the former group are less active than normally. Another fact to be considered is the normal variation in weight of the same muscle groups of opposite extremities. Studies by Lipschutz and Audova (5) on normal rabbits indicate that this variation is slight.

Histologic studies established the fact that this type of atrophy is associated with very simple structural changes. The loss in muscle bulk can apparently be accounted for on the basis of a diminished quantity of sarcoplasm in the individual muscle fibers. It is quite likely that slight changes also occur in the myofibrillar elements. If so, the changes are not revealed by the usual staining methods. The atrophied muscle fibers appeared to be packed more closely together, so that in a microscopic field of a definite area, a larger number of fibers was seen than in a corresponding area of normal muscle. There was no evidence of degeneration of the

muscle fibers, nor were there any of the usual features indicating an attempt at regeneration so characteristic of neurogenic atrophies.

Chemical analyses for water and nitrogen showed no significant change in water content between the atrophic and the normal muscle. The differences were small and occurred in both directions. This makes it highly improbable that there was any change in the degree of hydration of the protein. The quantitative decrease in protein content of this type of atrophy is associated with a decrease in the weight of the total muscle bulk, so that the relative proportion remains the same as that of normal muscle.

Physiologic tests likewise gave evidence of the absence of any degenerative process. Faradic and galvanic stimulation produced responses similar to those obtained in normal muscle.

Thompson, using rabbits, reported degeneration of muscle fibers with fibrosis, after six weeks' immobilization. He commented, however, upon the occurrence of circulatory and pressure complications in his experiment. This, no doubt, also accounted for the very rapid wasting which occurred. Froeboese, likewise, obtained degeneration of muscle fibers and replacement with fibrous tissue. Edema and pressure from the cast, no doubt, were responsible for these changes. It has been demonstrated by the present study that when such complications are avoided, the inactivity of muscle, such as is obtained by immobilization, does not give rise to any degeneration of muscle tissue.

Davenport and Ranson (6) studied the changes in skeletal muscle following tenotomy. After a period of from five to eight days, contracture occurred with a 20 per cent loss of weight. There was no degeneration of muscle fibers, nor increase in nuclei. They found an increase in the diameter of the fibers, more pronounced longitudinal striations, and blurred and wavy cross striations, which they considered characteristic of myostatic contracture. Such muscles, however, have been deprived of their "stretch reflex," and, hence, an additional factor has been added to that of simple inactivity. This, no doubt, accounts for the very rapid rate of atrophy resembling that which results following nerve section.

Lippman and Selig (4) found only a slight amount of muscle atrophy following fixation of the limb of the rabbit. They stated that such atrophy is not appreciable before the lapse of at least a month. Our results, however, imply a more rapid rate of atrophy, which corresponds to that commonly observed clinically.

In order to ascertain the status of the anterior horn cells which subserve the skeletal musculature subjected to inactivity, one monkey was treated as follows:

The left upper extremity was immobilized in a plaster of Paris cast for a period of one year. During this period the cast was removed at three-

month intervals to determine the state of the immobilized extremity, particularly to insure the avoidance of ischemic and pressure complications. Histologic examination of the atrophied muscles showed simple changes similar to those reported above. The cervical enlargement of the spinal cord and the peripheral nerves derived therefrom were examined. Employing hematoxylin and eosin and the Davenport silver stain, no difference could be demonstrated between cells of the right and left anterior cornu of the spinal cord. The peripheral nerves were likewise normal in appearance.

The factor of disuse has been mentioned by some in explanation of the changes observed in the anterior horn cells subserving extremities which have been amputated (Spatz, 7). These changes, designated as "axonal chromatolysis," have been considered retrograde and consist of a displacement of the nucleus to the axon hillock and a clumping of the Nissl bodies in that locality with a paling of the remaining cytoplasm. Other authors attribute these changes to injury to the axons.

The results of our experiment indicate that such muscular inactivity as is obtained by immobilization does not lead to demonstrable changes in the anterior horn cells of origin of the respective nerve supply. This is further supported by the lack of any changes in the intramuscular motor nerve endings.

CONCLUSIONS

1. Disuse atrophy is a distinct entity and is simple in character, as revealed by histologic findings.

2. Disuse atrophy consists primarily of a uniform reduction of the bulk of each muscle cell, especially of the sarcoplasm. It is not attended by any evidence of degeneration or attempts at regeneration. Irritability to electrical stimuli remains unaffected.

3. There is no alteration in the proportions of water and nitrogen content.

4. Simple disuse is not associated with any demonstrable changes in the anterior horn cells of origin of the respective nerve supply, and the atrophy of the peripheral musculature due to disuse does not result in such changes.

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THE EFFECT OF METHYLENE BLUE, CYSTINE AND CYSTEINE ON THE METABOLISM OF THE INTACT ANIMAL[1]

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It is well known that the foodstuffs which may be oxidized readily by the body are very resistant to oxidation in the test tube; hence the hypothesis arose that either the oxygen, or the food substrate, or both, were activated by enzymes in the body tissues. Warburg (1928, 1930) emphasized the activation of oxygen, and noted especially the significance of the catalysis by heavy metals; Wieland (1922, 1931), Thunberg (1930) and others presented evidence of the part played by the activation of the hydrogen atoms of the substrate. Later research on these problems demonstrated that both mechanisms were of importance in the oxidation of foodstuffs (Keilin, 1929, 1930; Barron and Hastings, 1933).

Other investigators have demonstrated that the addition of redox substances to tissues increased the rate of dehydrogenation of various substrates (Barron and Harrop, 1928; Barron, 1930; Wendel, 1933). The fact that the velocity of this reaction was increased by the addition of a hydrogen transporter indicated that more hydrogen might have been activated by the tissue dehydrogenases than could be taken up by the hydrogen transporters already present in the system. We have therefore investigated the effects of the addition of exogenous redox substances both in intact animals and isolated tissues.

EXPERIMENTS ON THE INTACT ORGANISM. The first group of observations were obtained on normal rats. Analyses of O_2 and CO_2 exchange were made in the Haldane (1892) open circuit respiratory chamber. The rats were usually fasted for 24 hours, and a determination of the respiratory exchange made. They were then injected with 0.5 cc. of 0.01 M methylene blue per 100 grams of body weight. The O_2 consumption and CO_2 production were subsequently determined during successive periods for 45 hours. The data obtained on 8 rats are presented in table 1.

It may be observed that the injection of methylene blue resulted in a depression of the R.Q. to levels as low as 0.55 in one case, and below 0.70 in almost all cases.

[1] Preliminary Reports, Proc. Soc. Exper. Biol. and Med. **30:** 906, 1933; This Journal **113:** 51, 1935.

This regular depression of the R.Q. followed by a rise is not to be observed in the control animals which were also fasted for twenty-four hours before observations were made but were not injected with methylene blue.

TABLE 1

Effect of methylene blue on the metabolism of normal rats

PERIODS	ANIMAL 1	ANIMAL 2	ANIMAL 3	ANIMAL 4	ANIMAL 5	ANIMAL 6	ANIMAL 7	ANIMAL 8
hours								
Foreperiod	0.69	0.76	0.73	0.72	0.72	0.77	0.70	0.90
0– 5	0.63	0.67	0.68	0.69	0.57	0.68	0.64	0.82
5–10	0.67		0.72	0.55	0.59	0.71	0.64	0.73
10–15	0.73	0.78	0.73			0.70	0.66	
15–20	0.80	0.84	0.72				0.73	0.69
20–25	0.82		0.72	0.60	0.61	0.69		0.84
25–30	0.75	0.70		0.79	0.83	0.81	0.78	0.71
30–35	0.77	0.72		0.75	0.82	0.73	0.74	
35–40	0.76		0.71					0.74
40–45	0.74		0.70	0.73	0.68		0.71	
Total R.Q.....	0.737	0.741	0.711	0.670	0.683	0.711	0.710	

TABLE 1A

Control observations on fasted rats

PERIODS	ANIMAL 1	ANIMAL 2	ANIMAL 3
hours			
0– 5	0.72	0.67	0.73
5–10	0.73	0.69	0.74
10–15	0.73	0.71	0.74
15–20	0.74	0.72	0.75
20–25	0.74	0.74	0.76
25–30	0.73	0.72	0.75
30–35	0.72	0.69	0.73
35–40	0.73	0.70	0.74
40–45	0.76	0.74	0.76
45–50	0.74	0.72	0.76
50–55	0.73	0.68	0.76
55–60	0.73		0.74
60–65	0.71		0.70
65–70	0.70		0.66
Average	0.729	0.707	0.737

The R.Qs. of table 1A reveal the small variations from the level of fat combustion.

On the other hand, in a number of instances, namely, experiments 1, 4, and 5 of table 1, the quotients were lower than could be explained by any

known type of physiological oxidation. This decrease in the quotient lasted in most cases from 10 to 20 hours. It was followed by a secondary rise which usually exceeded the quotient of the foreperiod. The R.Q. then fell gradually to the postabsorptive level. In those instances in which the R.Q. of the foreperiod approximated that attained during fasting, the quotient for the entire experiment was also found to be close to 0.70. The average quotient of these experiments was 0.709 ±0.009.

The fact that the R.Q. of the entire experiment approximated that of the fasted animal indicated that the metabolic energy was obtained from a food mixture similar to that oxidized during starvation. Such a food mixture would consist of fat, protein, and minimal amounts of carbohydrate or lactic acid. It was of interest, however, to investigate the intermediary

TABLE 2

Effect of methylene blue on the metabolism of phlorhizinized rats

PERIODS	ANIMAL 1	ANIMAL 2	ANIMAL 3	ANIMAL 4	ANIMAL 5
hours					
Foreperiod	0.69	0.72	0.73	0.72	0.68
0–10	0.63	0.62	0.68	0.63	0.63
10–20	0.71	0.66	0.65	0.55	0.58
20–30	0.65	0.66	0.54	0.60	0.60
30–40	0.64	0.71	0.56	0.57	0.53
40–50	0.74	0.78	0.65	0.68	0.68
50–60	0.83	0.83	1.10	0.88	1.05
60–70	0.82	0.80	1.13	0.88	1.12
70–80	0.70	0.67	0.99	0.86	1.05
80–90			0.84	0.86	0.94
90–100			0.71	0.68	0.69
Total R.Q	0.702	0.705	0.730	0.680	0.710

processes involved which resulted in such peculiar variations of the quotient. The first possibility that suggested itself was that methylene blue might cause a conversion of fat to carbohydrate, and therefore a depression of the R.Q. below 0.70. The secondary rise of the quotient seemed to follow the appearance of the methylene blue in the urine, and may have resulted from the oxidation of the newly formed carbohydrate.

The net result of these intermediary reactions, namely, the conversion of fat to carbohydrate plus the oxidation of the carbohydrate, would yield a R.Q. of 0.707 since the substrate oxidized was fat. To test this possibility a similar group of experiments were performed on phlorhizinized rats. In the phlorhizinized rat the renal threshold for glucose is reduced. Any carbohydrate formed from fat would be excreted and the secondary rise of the R.Q. should, therefore, fail to appear. The rats were fasted for three

days and injected with 20 mgm. of phlorhizin per 100 grams of body weight per day. We have previously noted a marked excretion of glucose in rats injected with a slightly smaller dose of phlorhizin (Goldfarb, Barker and Himwich, 1934). Metabolism determinations were made before and after the injection of 0.5 cc. of 0.01 M methylene blue per 100 grams of body weight. The results are summarized in table 2.

It may be seen that the data obtained closely resembled those of the animals receiving no phlorhizin. The original R.Q. was close to that characteristic of the post-absorptive condition. The first change due to the injection of methylene blue was a depression of the quotient below the normal physiological range. This was followed by a secondary rise which exceeded the original quotient in all cases. The R.Q. then gradually returned to the fasting level. The quotients obtained for the entire experiment varied from 0.670 to 0.730 with an average of 0.705 ± 0.006. The secondary increase of the R.Q. could not be explained by the oxidation of glucose since this substance is readily excreted by the phlorhizinized animal. It therefore seems probable that there was no conversion of fat to carbohydrate.

A second hypothesis suggested which might account for these variations of the R.Q. depends on the fact that methylene blue is an oxidation-reduction substance. We therefore investigated the effects of two other substances which are known to function as the effective portion of a redox system in the body (Hopkins, 1921), namely, cystine $\leftrightarrows$ cysteine. The method was the same as that used above. Five rats were injected with 1 cc. of 0.01 M cysteine per 100 grams of weight, and 5 others received 0.5 cc. of 0.01 M cystine per 100 grams of weight. The data are presented in table 3.

It was again observed that the quotients were depressed in the early hours, subsequently rose above the original R.Q. and then gradually returned to the fasting level. In 9 of these experiments in which the original quotient was approximately that obtained under fasting conditions, the quotient for the entire experiment approached 0.70. The average quotient in these animals was 0.709 ± 0.006.

The changes in the R.Q. following the injection of methylene blue, cystine or cysteine may have been due either to changes in the O_2 consumption, or CO_2 production, or both. In the present experiments the variations were found to result more from changes in the O_2 than the CO_2. The relationship between the R.Q. and the O_2 consumption (table 4) demonstrates that the low R.Qs. are associated with high O_2 consumption, and vice versa.

Discussion. In the present work we have investigated the effects of the addition of hydrogen transporters to the oxidative systems in the intact animal. The injection of methylene blue, cystine or cysteine into

rats has been found to produce similar changes in respiratory metabolism. These substances first caused a fall of the R.Q. below the levels which might be expected from any known form of physiological oxidation. Subse-

TABLE 3

Effect of cysteine and cystine on the metabolism of normal rats

1 cc. 0.01 M cysteine per 100 grams						0.5 cc. 0.01 M cystine per 100 grams					
Periods	Animal 1	Animal 2	Animal 3	Animal 4	Animal 5	Periods	Animal 1	Animal 2	Animal 3	Animal 4	Animal 5
hours											
Foreperiod	0.72	0.74	0.74	0.73	0.79	Foreperiod	0.77	0.67	0.71	0.76	0.68
0–11	0.68	0.73	0.73	0.64	0.76	0– 5			0.68	0.66	0.64
11–24	0.69	0.71			0.74	5–10	0.75	0.65	0.71	0.69	0.69
24–30	0.64	0.69				10–13	0.73	0.62			
30–37	0.67	0.68	0.65	0.61	0.75	13–24	0.63	0.66	0.67	0.69	0.71
37–48	0.63	0.63	0.73	0.72	0.75	24–30	0.82	0.72	0.71	0.72	0.71
48–55	0.67	0.73			0.80	30–33	0.74	0.85			
55–61			0.79	0.72		33–38	0.74	0.80	0.73	0.73	0.68
61–72	0.77	0.81	0.93	0.75	0.77	38–47	0.73	0.80	0.69	0.72	0.69
72–84	0.87	0.81	0.72	0.76	0.73	47–52		0.73			
84–95	0.79	0.77		0.80	0.72	52–59			0.72	0.71	0.70
95–105	0.72	0.77		0.69							
105–120	0.66	0.69									
Average. .	0.707	0.730	0.727	0.700			0.720	0.712	0.696	0.701	0.694

TABLE 4

Relation between R.Q. and O_2 consumption

R.Q.	Oxygen consumption		
	+*	0	−
0.51–0.60	12	0	0
0.61–0.65	19	2	1
0.66–0.70	27	3	19
0.71–0.75	26	2	27
0.76–0.80	4	5	12
0.81–0.90	0	0	18
0.91–1.0	0	0	2
1.01–1.10	0	0	4

Coefficient correlation—(-0.646 ± 0.027).

* +: higher than average for entire experiment. 0: equal to average for entire experiment. −: less than average for entire experiment.

quently the quotients rose above those of the foreperiod, and then gradually fell to the fasting level. Hawley, Johnson and Murlin (1933) have calculated the total fall in R.Q. which might result from an abnormal protein

metabolism, plus the conversion of glycerol to fat, and the development of ketosis. The combined correction for these three processes was 0.04. Since the fall in the quotient in the present experiments far exceeded this value, some other explanation must be sought.

The changes of the R.Q. were not due to the conversion of fat to carbohydrate since they occurred in the phlorhizinized animal. Such a conclusion is in harmony with the great mass of evidence which has failed to demonstrate the conversion of fat to carbohydrate in the mammalian organism (Lusk, 1928; Dann, 1933).

It was noted that in the experiments in which the R.Q. of the foreperiod approached 0.70, the quotients for the entire experiment also approximated 0.70. This indicated that the energy for metabolism during the entire experiment was derived from the complete oxidation of a food mixture which resembled that of fasting conditions. The mechanism of oxidation, however, evidently took place in two steps, the first of which resulted in a depression of the R.Q., while the second phase caused a rise of the quotient. In order to explain these results, it is suggested that the addition of the oxidation-reduction system facilitated the removal of H_2 from some substrate which was activated by tissue dehydrogenases. The reduced redox substance might then be readily oxidized by O_2 to yield H_2O plus the dehydrogenated form of the added substance. This reaction would result in the consumption of O_2 without the simultaneous production of CO_2 and the R.Q. would fall.

In the experiments with methylene blue the dye was observed in the urine during the first 24 hours. The secondary rise of the quotient occurred after the excretion of the dye. We have no data on the fate of the injected cystine or cysteine, but some estimate of the rate of excretion may be obtained from the work of Lewis and his collaborators. Lewis and Root (1922) recovered 73 per cent of the S of ingested cystine in the first 24 hour urine specimen. Lewis et al. (1924) also found that the administration of phenyluraminocystine to rabbits resulted in the conversion of an average of 50 per cent of the recovered compound into the cysteine derivative. It therefore seemed probable that the onset of secondary rise of the quotient in our experiments corresponded approximately with the disappearance of the hydrogen transporters from the body. If the fall of the R.Q. following the injection of a hydrogen transporting system is due to an increased velocity of dehydrogenation of some food substrate, the removal of the stimulus ought to result in a cessation of the process with a return of the quotient to the normal fasting level. However, instead of returning to post-absorptive values, the quotient reached far higher ones. This secondary rise of the R.Q. may be due to one or both of two intermediary reactions; the carbon rich residues might be oxidized and yield a high quotient, or the dehydrogenated compounds might be reduced

by accepting hydrogen. Either of these processes, or both combined, would result in the rise of the R.Q. observed. Michaelis and Smythe (1936) recently suggested that methylene blue inactivates carboxylase. Such an effect of methylene blue *in vivo* would diminish the formation of carbon dioxide and may explain in part the method of production of the carbon rich residues.

SUMMARY AND CONCLUSIONS

The respiratory exchange of fasted rats was studied after the injection of methylene blue, cysteine and cystine. The changes of R.Q. with each of these substances were similar. The R.Q. was depressed during the early hours, subsequently rose above the original quotient, and then returned to the post-absorptive levels. The results are best explicable on the basis of an increased velocity of dehydrogenation of some food substrate during the early hours of the experiment with the resulting formation of water. The secondary rise of the respiratory quotients was due either to the complete oxidation of the carbon rich residues, or the reconversion to their original form.

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STRYCHNINE AND THE CHRONAXIE

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The studies of the Lapicques on the disturbance of conduction between motor nerve and striated muscle, the phenomenon of curarization, included the block produced by strychnine (1). They found that administration of strychnine to the whole frog or application to the isolated nerve and muscle while not changing the chronaxie of muscle reduced that of the nerve, and they interpreted this as an "acceleration of the process of excitation." This phenomenon they used in support of an hypothesis of isochronism of muscle and nerve, and a general theory of curarization, as it supplied a method of production of heterochronism alternative to that which they had found with curare. In addition, they stated that when block occurred with strychnine the chronaxie of nerve had fallen to one-half its original value, thus the degree of heterochronism, a 1 to 2 ratio, was the same as seen with curare.

These results were confirmed by Bremer and Rylant (2) and extended by them in the development of a theory of the mode of action of strychnine on the central nervous system. The beliefs of the Lapicques have recently been reaffirmed (3). Still, it appeared that the action of strychnine would bear reëxamination.

EXPERIMENTAL. The procedure was identical with that used in a study of the action of narcotics on nerve (4). The cord-sciatic-gastrocnemius preparation of the Louisiana bull-frog was mounted in a paraffin chamber with two pairs of silver-silver chloride electrodes in Ringer's solution (NaCl 6.5, KCl 0.14, $CaCl_2$ 0.12, $NaHCO_3$ 0.2, NaH_2PO_4 0.01 gram per liter) and left for at least two hours before applying the drug, strychnine sulfate in this saline solution. The voltage-capacity relationship was determined with a condenser apparatus as used previously (4). Eighteen experiments were done, with application of strychnine either to the nerve at the cathode of the peripheral electrodes, or to this point and the muscle. The results of a typical experiment are shown in figure 1. The change in excitability, reversed by removal of the strychnine, was seen only at the peripheral pair of electrodes at which point the drug was

applied to the nerve. As this did occur without change at the central electrodes, it was an alteration in excitability of the fibers originally responding.

With concentrations below 0.025 per cent there was no discernible change in the excitability of the nerve, but at this concentration the typical effect as shown in figure 1 appeared and increased with increase in concentration until at 0.25 per cent applied to the nerve alone block occasionally occurred within three hours. With application to the muscle, a concentration of 0.05 per cent produced loss of indirect excitability within four hours; sooner with higher concentrations. Response of the muscle to direct stimulation was not lost with a concentration of 0.25 per cent.

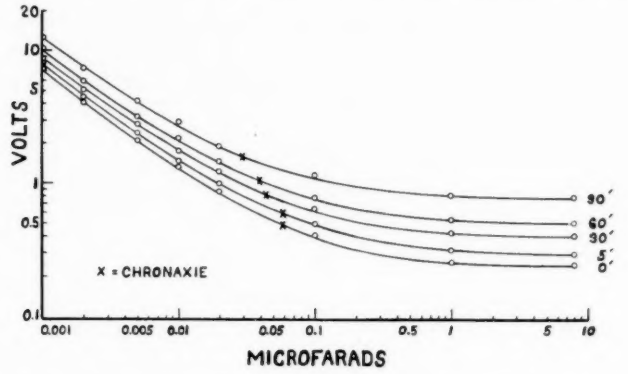

Fig. 1. (7/24/35) One-tenth per cent strychnine sulfate applied to muscle and to nerve at cathode of peripheral pair of electrodes. Determinations of excitability at 15, 45, 75, and 105 minutes are not shown. Loss of excitability at 120 minutes.

No attempt was made in these experiments to avoid the possible intervention of an *alpha* effect from the use of electrodes in a fluid bath (Lapicque); as in the previous study (4) no evidence was obtained of this dubious phenomenon having any influence on the results.

Discussion. The action of strychnine on the nerve fiber is not one of augmentation of excitability, as was concluded by the Lapicques, for it is indistinguishable from that of typical narcotics. Although the chronaxie is reduced, the *temps utile* is lengthened, the rheobase much increased. If excitability is defined in terms of the quantity or the energy of the electrical stimulus, as for instance with the formula of Lassalle (5):

$$\text{Excitability} = \frac{1}{(\text{Rheobase})^2 \times \text{Chronaxie}}$$

the effect of strychnine is clearly seen to be one of reduction of excitability.

In general, the evidence of others indicates a purely depressant action of strychnine on peripheral nerve fibers. Biberfeld (6) found only depression, and Danilewsky and Perichanjanz (7) claimed stimulation with low concentrations, but these early experiments were technically inadequate. Bronk (8) found chiefly a depressant action on sensory end organs in frog muscle and skin. Cowan and Ing (9) have shown that 0.01 molar strychnine hydrochloride (0.01 molar strychnine sulfate = 0.8 per cent) completely blocks the unmedullated nerve of *Maia squinado* in thirty minutes.

In the papers of the Lapicques, much is made of the fact that conduction between nerve and muscle failed when the chronaxie of the nerve had fallen to one-half its original value. This information is of questionable value as the excitability that is measured shortly before total failure of conduction is not that of the nerve fibers originally responsive to the minimal stimuli. This is shown by the rise in threshold that occurs at the central electrodes shortly before complete block takes place, indicating that the most sensitive fibers stimulated at first now fail to excite the muscle fibers they innervate. In these experiments no constancy of reduction in chronaxie at the time of block was observed. In table 1 is shown the percentage of normal to which the chronaxie had fallen at a time shortly preceding block, and the time at which block took place. The obvious relationship indicates that in the longer time required for the production of block, the greater has been the depression of the nerve at the stimulating electrodes. It would suggest that the diffusion of the agent into nerve is regular, that into muscle very irregular.

TABLE 1

CONCENTRATION OF STRYCHNINE	TIME OF BLOCK	CHRONAXIE: PER CENT OF NORMAL AT TIME OF BLOCK
per cent	*minutes*	*per cent*
0.10	120	53
0.25	105	67
0.25	90	68
0.25	75	75
0.25	60	86
0.25	40	95

As strychnine can interrupt conduction when applied to the nerve fiber alone, it is likely that this process sufficiently accounts for the failure of indirect excitability of muscle. The difference between the concentration required to block the nerve trunk and that required to interrupt neuro-muscular conduction may be due to the generally assumed high susceptibility of the terminations of the nerve fibers.

It is a pleasure to acknowledge the criticisms and advice of Dr. Hallowell Davis and the loan of apparatus by Dr. J. M. D. Olmsted.

SUMMARY

The action of strychnine on the motor nerve fiber is purely one of depression. The fall in chronaxie which occurs does not mean an augmentation of excitability as the Lapicques believed, and their conclusion that strychnine interrupts conduction between nerve and muscle by producing a heterochronism is unjustified.

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THE RELATIONSHIP OF THE SYNTHETIC MALE HORMONE, ANDROSTENDION, TO THE PROTEIN AND ENERGY METABOLISM OF CASTRATE DOGS, AND THE PROTEIN METABOLISM OF A NORMAL DOG[1]

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A previous report (Kochakian and Murlin, 1) from this laboratory has indicated that male hormone prepared from urine has no significant effect on the energy metabolism of castrate dogs whether administered in large single doses or in smaller doses over a period of time. A slight increase of about 10 per cent was noted in the case of a fat castrate dog after many repeated injections, but this effect was not considered very significant. In this same study it was noted that in every case there was a significant decrease in protein catabolism which was accounted for by a decrease in the urea fraction of the urine. The remaining constituents of urine and the nitrogen of the feces remained constant. It was also noted that during the repeated injections the decreased nitrogen catabolism attained a maximum within five days and further injections, or increasing the daily dosage, did not further increase the nitrogen retention, but only maintained it at the maximum.

In the meantime, the rapid advance in the chemistry of the male hormones has made available in pure crystalline form several interesting compounds related to the male hormones. Of these compounds, the synthetic male hormone, androstendion, was of particular interest to our study because of its midway position between the urinary products, which we have already studied, and the testicular product, testosterone, which we propose to study later. Androstendion possesses the α,β unsaturated ketone group of testosterone and the ketone group on the 5-membered ring of the urinary products, dehydroandrosterone and androsterone. Its potency on the capon's comb is equivalent to that of androsterone (2) (3) which is 3 to 4 times that of dehydroandrosterone (4), and only one-seventh

[1] This investigation was aided by a grant from the Committee on Scientific Research of the American Medical Association.

[2] This publication is taken from a thesis presented in partial fulfillment of the requirements for the degree Doctor of Philosophy, University of Rochester, June 1936.

that of testosterone (5), (6). Its action on the accessory sex organs lies midway between that of testosterone and androsterone and dehydroandrosterone (3). This unique biological and chemical midway position of androstendion, therefore, made an investigation of this compound of special interest to our studies.

Since the previous study showed that the same results were obtained on protein and energy metabolism by single large injections as by repeated smaller injections, which, however, produced a maximum in the protein retention within five days, it was felt that the purpose of this study could be most satisfactorily fulfilled by administering a large amount of hormone in three separate doses 24 hours apart. Thus the maximum effect on protein metabolism should be obtained and three instead of one experiment on energy metabolism made possible with more adequate recording of the same kind of results as are obtained by repeated injections. In order to eliminate the possibility that the decreased urea excretion might be due to retention of urea, the blood N.P.N. was determined. Also it seemed of interest to see if the rectal temperature of the dogs would be affected, since increase in body temperature is often associated with increased metabolism.

The retention of nitrogen noted in the previous report was attributed to regeneration of the accessory sex glands of the castrate animals. If this is the only use made of the nitrogen, then the fact noted that once a maximum retention of nitrogen was attained no further increase could be induced, either by further injections or by larger daily injections, would seem to indicate that a certain amount of hormone was required to maintain the physiological function of these glands at their maximum. It would follow, then, that a normal dog, producing his own male sex hormone to maintain the physiological functions of his accessory glands, would not react to the injections of male sex hormone as do the castrate dogs. Therefore, a normal dog was included in the protein metabolism phase of this study.

Preparation of androstendion. The preparation of androstendion was accomplished by means of the identical methods proposed almost simultaneously by Butenandt and Kudszuz (2) and Ruzicka and Wettstein (3). Two such preparations were made in our own laboratory for the purposes of this study. Preparation 1 was purified by repeated crystallization from dilute acetone. The final product, M.P. 164–5°C. (u.c.) was dissolved in olive oil so that 1 cc. of the oil solution was equivalent to 10 mgm. and was sterilized by passing through a Zeiss filter.[3] Preparation 2 was purified by recrystallizing once from dilute acetone followed by high vacuum distilla-

[3] We are indebted to Doctor Tittsler of the Department of Bacteriology for the sterilization.

tion[4] (7) and repeated recrystallization of the sublimate from dilute acetone. The pure product M.P. 164.5–165.5°C. (u.c.) was dissolved in olive oil so that 1 cc. of solution was equivalent to 15 mgm. of androstendion and was sterilized by autoclaving at 15 pounds pressure for 20 minutes.

The solutions of androstendion were assayed by the method employed in this laboratory (1): One capon unit[5] (C.U.) is the amount of hormone which, when injected daily on two successive days, will produce on the third or fourth day an average maximum increase of 3 to 4 mm. in length plus height (L + H) of the combs of at least three white leghorn capons. Nine capons were used for the assay and 1 mgm. of androstendion was found to be equivalent to 1.6–2.1 C.U.

Spectrophotometric studies[6] gave a typical α,β unsaturated ketone curve with a maximum absorption at 2360 Å.

TABLE 1
Daily diet

	DOG 1, 15 KGM. DOG 6, 13.1 KGM.			DOG 2, 26 KGM.		
	Grams	Grams N	Calories	Grams	Grams N	Calories
Beef heart	175	5.48*	434	225	6.89*	563
Cracker meal	60	1.20	233	80	1.60	322
Lard	30		270	40		360
Cod liver oil	5		45	5		45
Bone ash	10			10		
Wesson's salt mixture	3			3		
Totals		6.63	982		8.49	1,290

* Varied with each batch of beef heart.

Description. Three mongrel dogs were used. Dogs 1 and 2 were castrates that had been used before and are described in the previous report (1). Dog 6 was a normal adult dog.

Diets. The dogs were maintained on a beef-heart-cracker meal diet similar to that previously (1) described. This diet has now been used for 2 years and found adequate in all respects. The details of the diet are given in table 1. Dogs 1 and 6 were placed on the same intake.

[4] We wish to express our appreciation to Dr. Wm. M. Allen, Dr. W. H. Strain and Prof. W. R. Bloor for the use of their apparatus, and also to Doctor Allen for instruction in its proper use.

[5] Henceforth we shall use the term capon unit (C.U.) instead of bird unit (B.U.) which we used in previous publications. (Proc. Soc. Exper. Biol. Med. **32**: 1064, 1935; J. Nutrition **10**: 437, 1935). This change is desirable because we feel that the new unit is more descriptive and specific than the old.

[6] We are indebted to Dr. L. H. Stedman of the Department of Radiology for this study.

Procedure. The dogs were confined in metabolism cages and fed the prescribed diet at 5:30 p.m. Every other day catheterization was performed just before feeding. Two-day urine periods were employed except in cases noted in the tables. Total nitrogen of urine was determined by the usual Kjeldahl-Gunning method, urea plus ammonia by the Van Slyke-Cullen (8) aeration method and creatinine by the Folin (9) microchemical method. The ammonia was not determined separately since it had been shown to remain constant. The rest nitrogen was determined by difference. Creatine nitrogen was not determined, since in the previous report it was shown that any variations in creatine could not be considered significant because of the exogenous source of the creatine. Fecal nitrogen also was not determined because it had been shown to be constant.

The blood samples were taken from the jugular vein of dog 1 and the saphenous vein of dog 2. Potassium oxalate was used as the anti-coagulant. N.P.N. was determined on the Folin-Wu filtrate by Nesslerization (10). Blood urea was determined by the Leibhoff-Kahn (11) method.

The energy metabolism of dog 1 was determined by means of a Benedict closed circuit apparatus attached to an all-metal chamber, which was enclosed by a water jacket and contained thermometers reading to 0.02°C. at the air inlet, outlet and the top. The oxygen used was determined by passing through an accurately calibrated wet-test meter. Alcohol checks were made to ascertain the fitness of the apparatus. The average R.Q. of three such determinations was 0.663. Activity records were obtained by means of a kymograph.

"Helmet" for dog. The energy metabolism of dog 2 was obtained by the Tissot-Haldane method. An accurate spirometer was used for the collection of the expired air which was analyzed by means of a specially constructed modified Haldane apparatus of very good accuracy (12). The dog was under constant observation and respiration rates were repeatedly recorded. In the previous study the dog was connected to the spirometer by means of a face mask. Considerable difficulty was experienced in keeping the dog relaxed by means of this procedure. Therefore a Benedict type helmet (13) was constructed which proved very satisfactory. The bottom of an ether container 21 cm. by 16 cm. was removed and the ragged edges covered with adhesive tape. In the middle of the other end, the top, was soldered a 3 cm. x 4.5 cm. piece of brass tubing for an outlet to be connected to the flutter valve system of the spirometer. The dog's neck was shaved close up behind the ears and a dental dam neckpiece from a Benedict helmet was slipped over the dog's neck. The opening of this neck piece proved to be just the exact size for this dog. Then the can helmet was slipped over the dog's head and the dental dam was wrapped about the end of the can and held tightly there by means of a heavy elastic band. No sealing agent is needed about the dog's neck if the neck is kept well shaven and the proper size aperture is obtained in the dental dam.

TABLE 2

Blood chemistry

Experiment 1, dog 2

DATE	N.P.N.	UREA N	REST N
1936	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*
2/16	29.3	9.8	19.5
2/17	Injected 32–42 C.U. (20 mgm.) Androstendion, 10:19 a.m.		
	28.1	11.4	16.7
2/18	Injected 32–42 C.U. (20 mgm.) Androstendion, 9:24 a.m.		
	27.8	12.2	15.6
2/19	Injected 32–42 C.U. (20 mgm.) Androstendion, 10:15 a.m.		
	27.0	9.8	17.2
2/20	26.7	11.6	15.1
2/21	27.2	11.0	16.2
2/22	28.7	12.3	16.4
2/23	27.5	11.3	15.2
2/24	29.1	12.1	17.0

TABLE 3

Summary of nitrogen metabolism

Experiment 2, dog 2. Nitrogen intake, 8.49 grams N per day

DATE*	URINE CHEMISTRY – NITROGEN PER DAY				BLOOD CHEMISTRY		
	Total	Urea + NH_3	Creatinine	Rest	N.P.N.	Urea N	Rest N
1936	*grams*	*grams*	*grams*	*grams*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*
3/6					27.8	12.2	15.6
3/7					26.9	11.7	15.2
3/8							
3/9					26.4	11.3	15.1
3/10							
3/11	7.67	5.67†	0.238	1.76	27.3	12.3	15.0
3/12	Injected 96–126 C.U. (60 mgm.) Androstendion at 9:50 a.m.						
3/12					26.8	12.4	14.4
3/13	Injected 96–126 C.U. (60 mgm.) Androstendion at 9:45 a.m						
3/13	7.38	5.97	0.238	1.41	28.2	10.5	17.5
3/14	Injected 96–126 C U. (60 mgm.) Androstendion at 9:45 a.m.						
3/14					25.0	11.6	13.4
3/15	6.44	5.03	0.242	1.17	26.1	10.3	15.8
3/16					25.4	11.3	14.1
3/17	6.32	4.83	0.238	1.25	26.4	12.0	14.4
3/18					24.4	11.6	12.8
3/19	6.92	5.45	0.240	1.23	26.8	11.7	15.1
3/20							
3/21	7.08	5.64	0.248	1.19	26.9	12.5	14.4
3/22	7.29	5.66	0.240	1.29			

* End of urine period.

† Low value due to experimental error.

The dogs were thoroughly trained to their respective regimes. The surrounding temperature, which is recorded in the tables, was always

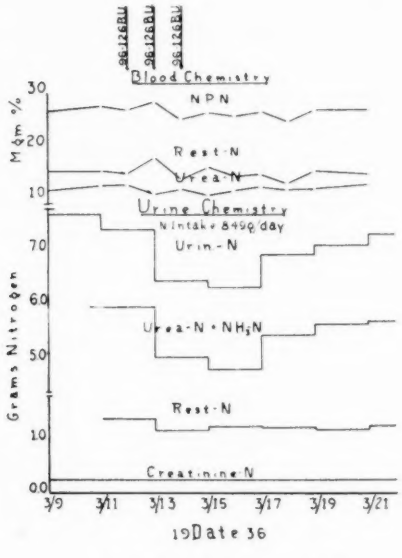

Fig. 1. Dog 2, experiment 2

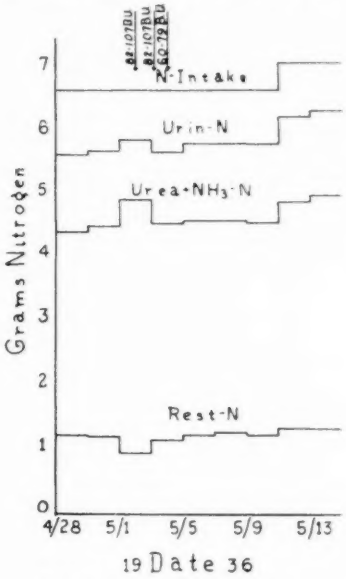

Fig. 2. Dog 6, experiment 1

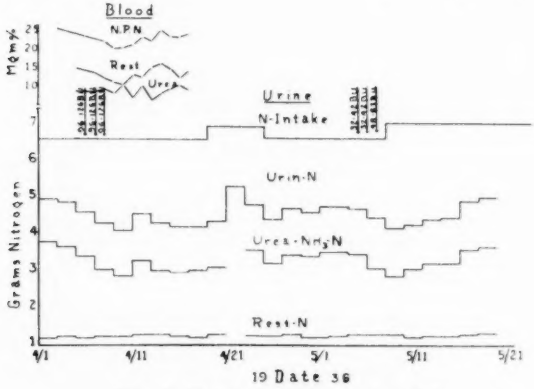

Fig. 3. Dog 1, experiments 1 and 2

within the normal range. The dogs were kept in the laboratory at all times.

The experiments on protein metabolism and energy metabolism were carried out simultaneously.

RESULTS. *Dog 2:* In experiment 1, table 2, only blood N.P.N. and blood urea were determined. The urinary nitrogen was not studied.

TABLE 4

Summary of nitrogen metabolism

Experiments 1 and 2, dog 1

DATE*	URINE CHEMISTRY—NITROGEN PER DAY				BLOOD CHEMISTRY		
	N-Intake	Total	Urea + NH_3	Rest	N.P.N.	Urea N	Rest N
1936	*grams*	*grams*	*grams*	*grams*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*
4/3	6.63	5.01	3.82	1.19	26.2		
4/5		4.93	3.69	1.26	25.0	9.3	15.7
4/6	Injected 96–126 C.U. Androstendion at 11 a.m.						
4/7		4.66	3.46	1.20	23.6	9.3	14.3
4/7	Injected 96–126 C.U. Androstendion at 11 a.m.						
4/8	Injected 96–126 C.U. Androstendion at 11:10 a.m.						
4/9		4.33	3 07	1.26	20.7	9.0	11.7
					21.0	11.2	10.8
4/11		4.15	2.91	1.24	22.0	7.7	14.3
					24.1	10.9	13.2
4/13		4.61	3.32	1.29	23.1	7.2	15.9
					25.9	8.8	17.1
4/15		4.34	3.06	1.28	24.0	9.9	15.9
					23.9	11.0	12.9
4/17		4.24	2.99	1.25	24.8	9.7	15.1
4/19		4.25	3.05	1.20			
4/21		4.40	3.12	1.28			
4/23	6.98	5.33					
4/25		4.86	3.61	1.25			
4/27		4.46	3.24	1.22			
4/29	6.67	4.76	3.47	1.29			
5/1†		4.63	3.43	1.20			
5/4		4.81	3.54	1.27			
5/5	Injected 32–42 C.U. Androstendion at 11 a.m.						
5/6		4.72	3.42	1.30			
5/6	Injected 32–42 C.U. Androstendion at 11:05 a.m.						
5/7	Injected 48–63 C.U. Androstendion at 11:15 a.m.						
5/8		4.48	3.18	1.30			
5/10		4.19	2.90	1.29			
5/12	7.09	4.29	3.09	1.20			
5/14		4.46	3.23	1.23			
5/16		4.48	3.24	1.24			
5/18		4.95	3.63	1.32			
5/20		5.04	3.70	1.34			

* Date at end of period.

† Three-day period.

In experiment 2, table 3, total nitrogen, urea plus ammonia nitrogen, creatinine nitrogen and by difference rest nitrogen of urine are each re-

corded. Blood N.P.N. urea nitrogen and by difference rest nitrogen are also recorded.

The urinary nitrogen drops after injections reaching a minimum sometime between the 1st and 3rd day after the last injection and then begins to return to normal. The urea plus ammonia nitrogen parallels, except in the 1st period, the drop in total urinary nitrogen, the creatinine remains absolutely constant and the rest nitrogen also remains constant. The first period shows a high rest nitrogen and a corresponding low urea and NH_3 nitrogen which is undoubtedly experimental error. The blood constituents remain fairly constant in both experiments. It can be said, therefore, that the drop in urinary nitrogen is not due to a retention of non-protein nitrogen constituents.

TABLE 5

Summary of daily maximum nitrogen retentions

DOG	HORMONE	WEIGHT	C.U. INJECTED PER DAY	AVERGE DAILY MAXIMUM N RETENTION	MAXIMUM N RETAINED PER KGM. PER DAY
		kgm.		*gram*	*gram*
1	Urinary	13	13–17*	0.6	0.05
	Urinary	13	25–34*	0.7	0.06
	Urinary	12	38–51*	0.6	0.05
	Urinary	12	25–34*	0.7	0.06
	Androstendion	15	96–126†	0.8	0.05
	Androstendion	15	32–42§	0.9	0.06
2	Urinary	22	25–34*	1.3	0.06
	Urinary	22	38–51*	1.3	0.06
	Urinary	22	13–17*	0.4	0.02
	Urinary	22	208–310‡	1.3	0.06
	Androstendion	26	96–126†	1.4	0.05

* Injected daily over a period of 15–25 days. Maximum values obtained after first 5-day period.

† This amount administered for 3 successive days.

‡ Single injection.

§ This amount administered for 2 successive days and on third day 48–63 C.U injected.

The graphical presentation of the urinary and blood constituents during experiment 2 are presented in fig. 1.

Dog 1: The results for this dog, table 4 and fig. 3, show the same general effect as for dog 2, a decrease in urinary nitrogen, which is accounted for by the urea phase, the rest nitrogen remaining constant. The blood N.P.N. shows a slight drop subsequent to injection.

In the first experiment, table 4, on this dog, the hormone was administered in identical amount and manner as in experiment 2 (table 3) of dog 2.

In the second experiment, table 4, which was made directly after the first, the hormone was administered in an identical manner as in the first experiment, but in smaller amounts. On the first and second day, 32 to 42 C.U. or one-third the previous amount, and on the third day 48 to 63 C.U. or one-half the previous amount were administered. The maximum nitrogen retained per day, however, in both cases, is about the same—0.8 gram for experiment 1 and 0.9 gram for experiment 2. There is, nevertheless, a noteworthy difference. In experiment 2, as in experiment 2, dog 2, there is an immediate gradual return to basal after the point of maximum nitrogen retention is attained, while in experiment 1 there is a prolonged period of about 8 days of maximum nitrogen retention.

TABLE 6

Summary of nitrogen metabolism

Experiment 1, dog 6

DATE AT END OF PERIOD	N-INTAKE	URIN. N	$\frac{N}{UREA + NH_3}$	REST N
1936	*grams per day*	*grams per day*	*grams per day*	*grams per day*
4/30	6.67	5.66	4.40	1.26
5/1		5.72	4.50	1.22
5/2	Injected 82–107 C.U. Androstendion at 9:15 a.m.			
5/3	Injected 82–107 C.U. Androstendion at 9:15 a.m.			
5/3		5.90	4.94	0.96
5/4	Injected 60–79 C.U. Androstendion at 10:30 a.m.			
5/5		5.68	4.53	1.15
5/7		5.85	4.58	1.27
5/9		5.86	4.57	1.31
5/11		5.82	4.55	1.27
5/13	7.09	6.26	4.89	1.37
5/15		6.33	4.98	1.35

A comparison of the daily maximum nitrogen retention by dogs 1 and 2, table 5, shows that the amount retained by dog 2 is much greater than that by dog 1, even when identical amounts of hormone are administered. If, however, these values are converted to a weight basis, this difference disappears. The amount retained is the same for both dogs per unit of body weight, 0.05 to 0.06 gram nitrogen per kilogram. This is supported by a similar analysis of the results obtained with the urinary extracts in the previous study (1). Dog 2, in this study, showed an average daily maximum nitrogen retention of 1.3 gram or 0.06 gram N per kgm. per day, whether receiving 25 to 34 C.U. per day or 38 to 51 C.U. per day for more than 5 days or a single injection of 208 to 310 C.U. With a dosage of 13 to 17 C.U. per day for over 5 days, this same dog showed a daily nitrogen retention of only 0.3 to 0.4 gram or 0.01 to 0.02 gram N per kgm. per day,

which is only about one-fourth of that retained as the result of a dose twice as great. The results for dog 1 showed a maximum daily nitrogen retention of 0.6 to 0.7 gram or 0.05 to 0.06 gram per kgm. per day, with the administration of 13 to 17 C.U., 25 to 34 C.U., or 38 to 51 C.U. per day for periods of 15 to 25 days. It is noteworthy that dog 1 showed no significant decrease in the amount retained with the smallest dose, 13 to 17 C.U. per day, in contrast to dog 2. This is probably due to a smaller requirement of hormone by dog 1, which is much smaller than dog 2. There is, therefore, not only a similar weight relationship of the maximum daily nitrogen retention for both dogs, but also a similar quantitative effect is produced with the two different types of hormones. Furthermore, it might be stated, with due allowance for the fact that the data to demonstrate it are meagre, that dog 1 reaches this maximum state with one-half the dose required by dog 2. Since dog 1 is also one-half as heavy as dog 2, it would follow that the daily maximum nitrogen retention is proportional to the C.U. per kgm. This should be substantiated by more evidence.

Dog 6: The results obtained in the case of this normal dog are in marked contrast to the consistent results obtained with the castrate dogs. This dog received three successive daily injections of hormone in doses slightly less than the larger dose but much greater than the smaller administered to the castrate dogs. There is no indication of any effect on the nitrogen excretion. In fact there is an apparent rise of about 0.1 to 0.2 gram N per day, but this quite obviously is not significant. Also there is an apparent increase of urea N with a consequent decrease of rest N in the first 2 days of injection, but this change is only a little greater than daily variation and therefore cannot be ascribed to the injections, for there is no further change in the same direction due to a third injection. In fact, the urea and NH_3-N and rest N are in the normal range again. These changes, therefore, must be attributed to experimental or metabolic variations. The apparent large increase of nitrogen excretion in the last two periods is due to an increased nitrogen intake caused by the use of a new batch of beef heart which had a higher percentage of nitrogen. These results are presented in table 6 and Fig. 2.

It must be said, therefore, that the administration of androstendion does not affect the protein metabolism of a normal dog.

Energy metabolism. Prior to carrying out the hormone experiments, a series of basal runs over a period of time was obtained to reaccustom the dogs to their respective regimes of metabolic determinations. Also just before each daily injection one or two metabolism periods were obtained followed by several more after the injection.

Although the protein metabolism was determined during all these experiments except experiment 1 on dog 2, no attempt is made to distribute the calories to the various constituents of fat, carbohydrate and protein.

We are primarily interested in noting whether there is any effect on the total energy metabolism.

Calories per square meter per hour are calculated according to the method of Cowgill and Drabkin (14).

Dog 1, whose metabolism was determined by means of a chamber at-

TABLE 7

Summary energy metabolism: Dog 1

DATE	NUMBER OF DETERMINATIONS	DURATION OF DETERMINATIONS	OBSERVED R.Q.	CALORIES PER HOUR	CALORIES PER SQUARE METER PER HOUR	AVERAGE TEMPERATURE OF CHAMBER	WEIGHT
1936						*°C.*	*kgm.*
3/31	2	10:29–12:18	0.77±0.01	18.7±1.0	30.2±1.6	23.5±0.1	15.2
4/1	2	10:03–12:08	0.76±0.01	18.8±1.3	30.3±2.0	22.5±0.1	
4/2	5	10:04– 2:58	0.77±0.01	18.7±0.7	30.1±1.1	22.0±0.5	
4/4	4	9:06– 1:05	0.79±0.01	18.1±1.2	29.2±2.0	22.7±0.2	15.0
4/6	2	9:00–10:53	0.77±0.04	18.6±1.2	30.0±2.0	21.2±0.1	
	Injected subcutaneously 96–126 C.U. (60 mgm.) Androstendion, 11:00						
	4	11:57– 4:40	0.77±0.03	20.0±0.6	32.2±1.0	22.4±0.4	
4/7	2	8:54–11:01	0.75±0.03	18.9±0.5	30.4±0.9	22.1±0.1	15.1
	Injected subcutaneously 96–126 C.U. (60 mgm.) Androstendion, 11 a.m.						
	4	12:22– 4:56	0.78±0.02	19.6±1.0	31.6±1.6	23.3±0.3	
4/8	1	9:04–10:03	0.73	18.6	30.0	23.2	16.0
	Injected subcutaneously 96–126 C.U. (60 mgm.) Androstendion, 11:10						
	4	12:31– 4:48	0.77±0.02	19.2±0.5	30.9±0.8	23.9±0.3	15.0
							15.4
4/9–4/27	13	9:00–12:00	0.76±0.02	18.5±0.4	29.8±0.7	23.5±0.5	15.5
5/1	2	9:27–11:21	0.74±0.01	18.5±0.4	29.8±0.7	24.5±0.1	15.4
5/4	2	9:38–11:42	0.75±0.01	18.7±0.5	30.1±0.8	23.6±0.1	
5/5	2	8:50–10:51	0.77±0.01	18.0±1.0	29.0±1.6	24.1±0.1	
	Injected 32–42 B.U. (20 mgm.) Androstendion, 11 a.m.						
	4	11:45– 4:17	0.80±0.01	18.0±0.8	29.0±1.2	24.4±0.2	
5/6	2	8:54–10:57	0.75±0.01	18.4±0.2	29.7±0.3	24.1±0.1	
	Injected subcutaneously 32–42 C.U. (20 mgm.) Androstendion 11:05 a.m.						
	4	12:07– 4:19	0.79±0.02	17.9±0.5	28.8±0.8	24.1±0.1	
	Injected subcutaneously 48–63 (30 mgm.) Androstendion, 11:15						
5/7	3	12:27– 3:53	0.75±0.03	18.6±0.9	30.0±1.5	24.5±0.5	

tached to a Benedict Universal apparatus, was injected with the prescribed dose after two successive periods of approximately 1 hour each were obtained, returned to his cage for a short rest period of approximately $\frac{1}{2}$ hour, placed again in the metabolism chamber and after a preliminary period of approximately $\frac{3}{4}$ hour, 4 successive determinations of approximately one hour each were made.

As can be seen from table 7, dog 1 shows no inclination to increase or decrease its energy metabolism, either immediately after injection or 24 hours after injection, nor is there any later effect due to the repeated

TABLE 8

Summary energy metabolism. Dog 2

Weight 26.0±0.1 kgm. Length 90 cm. Surface area 0.83 sq. m.

DATE	TIME BEGINNING OF PERIOD	ROOM TEMPERATURE	OBSERVED R.Q.	CALORIES		VENTILATION RATE	RECTAL TEMPERATURE	RESPIRATION
				Per hour	Per sq. m. per hour			
1936		*°C.*				*liters per hour*	*°C.*	
2/14–2/15	9:25– 2:14	24.1±0.4	0.73±1	25.3±0.3	30.4±0.4	170.2±9.4	39.0	14±2
2/17	10:01	23.4	0.73	25.5	30.7	175.6	39.1	14
	10:19	Injected subcutaneously 32–42 C.U. Adrostendion						
	1:00	23.7	0.71	25.9	31.2	180.1	39.0	14
	3:38	23.6	0.74	25.6	30.8	199.1	39.1	16
2/18	9:06	23.4	0.75	25.1	30.2	180.9	39.0	14
	9:24	Injected subcutaneously 32–42 C.U. Androstendion						
	10:31	23.8	0.74	25.5	30.7	178.6		14
	3:48	24.4	0.73	26.3	31.6	170.4	39.0	10
2/19	9:23	25.1	0.75	25.7	30.9	213.6	39.0	17
	10:15	Injected subcutaneously 32–42 C.U. Androstendion						
	11:41	24.3	0.72	24.1	29.0	163.0		14
	2:24	24.4	0.72	25.8	31.0	173.2	39.0	11
	4:55	24.8	0.75	25.9	31.2	197.3		14
2/20	9:50	25.2	0.74	25.3	30.4	178.2	39.0	12
2/21–2/24*	10:05–10:31	24.7±0.5	0.74±0.1	24.3±0.3	29.3±0.4	174.7±5.1	38.9	13±1
3/ 7–3/11†	8:28– 4:17	25.7±0.7	0.74±0.2	23.5±1.0	28.3±1.2	162.5±12.6		12±1
3/12	8:55	25.2	0.74	23.1	27.8	161.6		13
	9:50	Injected subcutaneously 96–126 C.U. Androstendion						
3/13‡	8:51– 9:10	24.5±0.0	0.73	23.7±0.1	28.5±1.2	142.0	38.3	10
	9:45	Injected 96–126 C.U. subcutaneously Androstendion						
	11:31	25.2	0.77	24.5	29.5	186.5	38.2	14
	2:07	25.8	0.74	22.2	26.7	138.4		11
	3:34	25.8	0.75	23.0	27.7	164.1		13
3/14	9:17	24.3	0.75	22.7	27.3	169.3	38.0	13
	9:45	Injected subcutaneously 96–126 C.U. Androstendion						
	1:09	25.1	0.78	23.0	27.7	165.6	38.1	13
	4:38	25.7	0.74	25.3	30.4	191.0		16
3/15–3/21§	9:36–10:50	24.4±0.4	0.74±0.01	23.4±0.9	28.1±1.1	161.1±5.7	38.0±0.1	13±1

* Average of 3 determinations.
† Average of 9 determinations.
‡ Average of 2 determinations.
§ Average of 4 determinations.

injections. The dog's weight shows a slight increase which probably is not significant.

The conditions during the entire experimental period were well con-

trolled. The metabolic determinations were made in as nearly equal periods of time as possible, the calorimeter chamber temperature was kept within favorable limits, in fact almost constant at 23 ± 1°C.

Dog 2: After a preliminary period of basals which indicated that the dog had become accustomed to his new helmet, the experiments were started. Prior to each injection a metabolic determination was obtained and after the injection 2 or 3 more determinations were obtained with the exception of the experiment on March 2, 1936. Subsequent determinations were not obtained on this day because the dog proved refractory at several attempts. The results, table 8, show no tendency for an increase in metabolism at any time during the entire experimental period. In fact, there is a slight drop of about 8 per cent between the first series of experiments, February 14 to February 24, and the second series, March 7 to March 21, which is accompanied by a similar drop in rectal temperature. This is difficult to attribute to the effects of the injections, but seems rather to be a result of a better adaptation of the dog to his new helmet as is evidenced by a lower rectal temperature.

Discussion. The results in this study are in complete confirmation of the results obtained in the previous study (1). It cannot be said, therefore, that there are any qualitative or quantitative differences between the urinary extracts of male hormone and the synthetic male hormone, androstendion, in their relation to the energy and protein metabolism of castrate dogs, although they are chemically different.

The failure to increase the maximum nitrogen retention with the administration of increased amounts of male hormone indicates that any male hormone administered beyond a certain dosage is unnecessary and wasteful. Apparently some controlling mechanism renders the excess male hormone unharmful. Fear of untoward effects from excessive doses of male hormone should therefore not obstruct the clinical application of this material.

The similar quantitative maximum daily nitrogen retention, obtained by a comparison of the results of this study on androstendion with the results of the previous study on the urinary hormone, was not anticipated. It was rather expected that the difference in the chemical properties of the materials used in the two studies would show a similar qualitative effect but a different quantitative effect. Ruzicka, Goldberg and Rosenberg (15) (see also Ruzicka and Wettstein, 6) in an analysis of the relationship of chemical structure of the various natural and synthetic male hormones, found that substances containing a keto group in the "three" position, as in androstendion, have a greater effect on the physiological reaction of the accessory sex organs of castrate rats, than do the substances containing a hydroxyl group in this position, as in the urinary product.

The results of the study of the blood N.P.N. which never showed an increase, and in fact showed a decrease of about 15 per cent (dog 1, experiment 1), indicate that the decrease in urinary nitrogen is not due to a retention of non-protein nitrogen constituents. It rather indicates that there is a utilization of circulating amino acids for rebuilding new tissue.

The interesting results obtained on the protein metabolism of the normal dog are worthy of remark. This dog with its testes intact is presumably producing enough male hormone to maintain its accessory sex organs in a normal physiological state. Not so the castrate dogs. When male hormone is administered to these dogs, a stimulus is provided for the restoration of the accessory sex organs which is met by a mobilization of the circulating amino acids to regenerate the atrophied organs. Such retention of protein beyond regeneration would seem to be confined to structures atrophied by castration for maintenance of protoplasm and production of their secretions. It appears also, that if the physiological requirements for male hormone are met, any excess male hormone will not be effective in producing further changes in protein metabolism. This hypothesis is further supported by the fact that in the previous study it was noted that the nitrogen retained reached a maximum within 5 days and that a further increase of the daily dose or the extension of the injections did not produce an increased nitrogen retention, but only maintained this maximum. This phenomenon is similar to that observed in the present study.

The male hormone illustrates well the action of a specific growth regulator. When present in adequate amount it induces anabolism of protein (nitrogen retention) in specific structures. Inherent limitations of growth, tracing back to the germinal chromosomes, however, prevent continuous uncontrolled expansion of these structures in proportion to the amount of hormone present. In all probability the full development of the several accessory sex glands under the influence of the animal's own normally produced hormone cannot be exceeded after castration under the effects of synthetic or extracted hormone. Excess growth beyond the normal limits would be teratological.

Relationship in regulatory effects between sex hormones and the growth hormone of the pituitary naturally are suggested, but this is a problem of itself. As a first step in the elucidation of this problem, experiments are now underway to find a maximum dosage, if any, beyond which there will be no further increase in the size of the accessory glands of castrate rats. Would the gonadotropic hormone from the anterior pituitary be able to condition the accessory structures for a larger effect? Many other questions can be raised for which there is yet no answer.

The energy metabolism in both castrate dogs remained constant within a maximum range of 10 per cent. The decreased caloric output due to

decreased protein metabolism was very probably compensated for by an increased fat or carbohydrate metabolism or of both. The decreased energy from protein, however, is not very great, a maximum of about 1.5 calories per hour for dog 2, for 1 or 2 days, and at all other times for both dogs it is much less. This decrease falls well within the range of experimental error.

The confirmation of the results obtained on energy metabolism with urinary extracts plus unpublished similar data on human subjects, leads only to one conclusion, namely, the endocrine function of the testes as represented by the male hormones cannot be considered as a rejuvenating mechanism in the calorigenic sense. In order to make this more conclusive, a similar study with testosterone will be undertaken.

SUMMARY

The results of injections of androstendion in castrate adult male dogs confirm the previous findings with the use of urinary male hormone extracts.

The protein metabolism decreases as a result of injection. The nitrogen retention, which is borne by the urea fraction of the urine, is attributed for the present to the regeneration of the accessory sex organs and possible production of their secretions.

The daily maximum nitrogen retained and the amount of male hormone necessary to produce this effect are proportional to the body weight and are the same for the urinary product and androstendion, although the two are chemically different.

The blood N.P.N. and urea show if anything a decrease, but never an increase.

The protein metabolism of a normal dog in contrast to the castrate dogs shows no effects.

It is suggested that there is a maximum physiological requirement of male hormone, beyond which additional hormone has no effect.

The energy metabolism of the castrate dogs is not affected. Thus further evidence is provided that the male hormones cannot be considered as calorigenic agents.

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UREA CLEARANCE AND PROTEINURIA DURING EXERCISE

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The presence of increased amounts of protein, red cells and casts in the urine secreted during exercise has been frequently demonstrated. These findings, however, are not regarded with any serious apprehension when the kidney is known to function quite satisfactorily during normal conditions. The presence of albuminuria during exercise was probably first discovered by Leube (1) in 1878, although credit is given to De la Camp by Schmidt and Kohlrausch (2). A decrease in kidney function as measured by the urea clearance test has also been found during increased muscular activity, by Addis and Drury (3), Mackay (4), Van Slyke, Alving and Rose (5) and Benzinger (6). The purpose of the studies reported in this paper was to determine if possible any correlation between the degree of albuminuria and changes which might occur in the urea clearance during vigorous exercise.

Our subjects were all healthy young males attending the Lawrenceville School, ranging in age from 14 to 20 years. They lived under quite identical conditions, including diet. Three sports, football, soccer and basketball were chosen as the form of exercise, primarily because these games are popular with the normal boy; secondarily because regulation games are long enough to secure adequate specimens of urine for analysis and finally to ascertain any manifest difference between these three sports. All games were played according to interscholastic rules, supervised by competent officials. The existing rivalry was so intense as to force each boy to play to the limit of his endurance.

EXPERIMENTAL. At least one and frequently two boys from each team, playing in the most strenuous positions, were selected as subjects. Samples of blood and urine were discarded unless the subject played the full regulation game, and voided immediately after the cessation of exercise. Despite the fact that the sample of blood was collected and the urine discarded at the last possible moment before the game began, and another sample of blood obtained and the urine collection made immediately after the game in the gymnasium, actual exercise represents only about one-half of the collection time. The balance of the period was consumed by rest periods between quarters and halves and "times out."

Normal clearances for each subject were obtained in the morning in the routine procedure recommended by Möller, McIntosh and Van Slyke (7). Their breakfasts consisted of fruit, cereal and milk. They spent the interim between collections either in their rooms or attending classes. The two collection periods varied from three-quarters to one hour each.

The aeration method, using Squibb's urease was used to determine the blood and urine urea nitrogens. The protein in the urine was determined by Folin's (8) quantitative method. All samples were analyzed in duplicate.

Results. Complete studies were carried out on twenty-nine boys, eleven subjects playing football, six soccer, and twelve basketball. McIntosh, Möller and Van Slyke's (9) line chart was used to correct for the age and height of the subjects. All clearance values were calculated as per cent of normal. Minute volumes of urine below 2 cc. per minute were computed as standard clearances, volumes above this figure as maximum.

The average normal clearance for the entire group of subjects was 136.4 per cent with a standard deviation of 43 and a standard error of the mean at ± 8. With the normal value placed at 100 by Van Slyke, this age group appears to have higher clearance values than those for adults and the lower age group reported by Cullen, Nelson and Holmes (10). The lowest normal clearance value obtained was 85 per cent, the highest 241 per cent. The blood urea nitrogen averaged 11.4 mgm. per 100 cc. of blood, ranging from a minimum of 6.1 mgm. to a maximum of 15.4 mgm.

During exercise, the group playing football cleared 47.5 per cent of their normal, soccer 53.3 per cent and basketball 37.3 per cent. Despite the greater percentage decrease in the clearance found in the group playing basketball as compared to the groups playing soccer and football, the results when analyzed statistically show the standard errors of the various means of the three groups to be such that would indicate that there is no significant difference between them. The average blood urea nitrogen increased from 8.9 mgm. to 10.6 mgm. per 100 cc. during football, 12.1 mgm. to 12.5 mgm. during soccer and 12.8 mgm. to 13.3 mgm. during basketball.

The amounts of albumen excreted by the different subjects in the various sports, computed on the basis of half-hour periods, varied tremendously. The type of sport did not appear to make any essential difference. No correlation appeared to exist between the percentage drop from the individual's normal clearance and the degree of proteinuria. A careful study of the posture, normal blood pressure, deviation from the normal weight for age and height for each subject, failed to offer any solution for the widely different amounts of albumen excreted and computed for half-hour periods.

The average urine minute volumes during the periods of exercise re-

mained remarkably constant, being 0.43 cc. per minute for football, 0.51 cc. for soccer, and 0.46 cc. for basketball. Attempts to link the degree of proteinuria with the minute volumes failed completely.

Discussion. The definite decrease of the urea clearance in every one of our subjects is in accord with the findings of the previously mentioned investigators (3) (4) (5) (6). The failure to show any correlation between changes in urea clearance and proteinuria induced by vigorous exercise are interesting inasmuch as Medes and Berglund (11) were unable to correlate the proteinuria ensuing when subjects assumed the lordotic position with the concomitant changes in creatinine clearance.

The inconstancy of the proteinuria resulting from vigorous exercise has already been noted by Roberts (12). Our results not only corroborate his findings, but a series of unpublished estimations made from a group of subjects during each game of an entire season bears out this inconstancy. Neither the excitement before the game, the closeness of the match nor the minute volume of urine secretion seemed to play any part in the amount of protein excreted. In general, however, there was a gradual decrease in the amounts excreted as the seasons progressed, suggesting training and physical fitness as a possible contributing factor.

Hellebrandt, Walters and Miller (13) were unable to raise the urine minute volume during vigorous exercise, by increasing the fluid intake. We attempted this same procedure in a number of our subjects. Quite frequently the subjects flatly refused to drink more than their accustomed amounts. In a few instances, we were able to raise the fluid intake considerably. The minute volumes of this latter group, however, remained consistently low, and practically the same as those obtained from the rest of the group.

SUMMARY

1. A definite fall in urea clearance was noted in every one of our subjects engaged in playing regulation football, soccer and basketball games.

2. No correlation existed between the degree of proteinuria induced by exercise and the changes in the urea clearance values obtained during this same period.

3. With few exceptions, the urine minute volume is quite low and uniform for the three different sports.

4. There is no significant difference in the fall of the urea clearances obtained between the three sports.

5. The average normal clearances obtained from this age group (males 15–20 years) 136.4 per cent is definitely higher than that for adults or lower ages than our group.

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THE CONCENTRATION OF NUCLEATED CELLS IN THE BONE MARROW OF THE ALBINO RAT[1]

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In the study of the chronic toxicity of small amounts of materials such as lead and arsenic, a more delicate measure of the status of the bone marrow is needed. The detection of slight changes in histological sections of bone marrow is extremely difficult. A method of counting the total number of cells per cubic millimeter volume of marrow is essential for the detection of minimal toxic effects. Several methods (1), (2), (3), (4) are available for the differential classification and enumeration of the different types of cells present in the marrow. Of these methods the first (1) has proved the most satisfactory in this study. In this method cover slip films, prepared from a suspension of bone marrow in fresh blood serum of the same species of animal, are dried in air and stained with Wright's blood stain. The present paper is a report of the observations made on a series of normal animals with respect to the number of nucleated cells in the marrow at various age periods.

The animals used in this study were albino rats, stock animals of both sexes, and varied in age from 4 days to 13 months. All were of the Osborne-Mendel stock, although part had been raised in the Division of Pharmacology and part in Vitamin Division, where different stock diets were in use. No animals were included that were on inadequate diets or that had received any toxic substance. For counting, a modification of the method employed by Isaacs (1) has been used on the marrow of the femur. Because of the absence of bone spicules, it is a relatively simple matter to remove the marrow, either for dilution in a blood-counting pipette or for fixation and section without decalcification. The details of this method, which has been developed and used as a rapid and routine autopsy procedure for the enumeration of the total number of nucleated cells per cubic millimeter of marrow, are presented in the next paragraph. The number of mature red cells has not been determined.

[1] This study is a part of a comprehensive investigation of the toxicity of small amounts of lead and arsenic which is being carried out by the Division of Pharmacology of the Food and Drug Administration under the direction of Dr. Erwin E. Nelson and Dr. Herbert O. Calvery.

[2] Associate Pharmacologist.

EXPERIMENTAL. The procedure which has been found satisfactory is as follows: the shaft of the femur is dissected free of muscle and the distal end of the bone cut off, exposing the marrow in the shaft. With the usual red-blood-cell-diluting pipette, marrow is aspirated up to the first 0.001 mark, and then diluted to the 1.01 mark with 1 per cent acetic acid. This diluted material is then shaken in a mechanical shaker for 30 minutes. The counting chamber used for blood cell counts is filled, a few minutes allowed for settling, and the cells in three of the 1-square-millimeter areas counted. With the average of these three squares, the thickness and dilution factor is 10,000.

With this procedure the values recorded in the chart were obtained

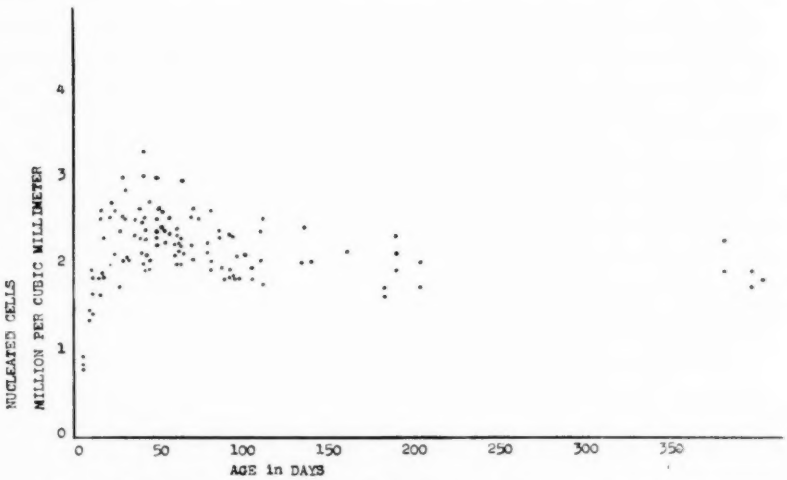

Fig. 1. The number of nucleated cells per cubic millimeter of the bone marrow of the femur of the white rat at different ages.

from 125 stock colony albino rats in which no abnormalities were found at autopsy. (See figure.) Up to about 3 weeks of age (23 animals), a progressive increase in the concentration of nucleated cells occurs. Between 3 weeks and 3 months, the average number per cubic millimeter of femoral marrow (70 rats) was 2.40 million with 84 per cent of the observations falling between 2.00 and 2.75. In observations on 32 rats from 3 months of age up to 13 months, the average count was 2.02 million. In this period there was qualitatively an increase in the amount of fat in the marrow.

DISCUSSION. The accuracy and validity of this technic were scrutinized from several points of view—namely, the uniformity of suspension in the

diluting pipette and the counting chamber, the adequacy of the size of the marrow sample employed, the agreement obtained in counts from both femurs and from different parts of the same femur. In many tests of these points the maximum single variation was 530,000 cells per cubic millimeter; most of the differences were of the order of 150,000 cells per cubic millimeter. In spite of the summation of errors that undoubtedly occurs, the agreement in counts is sometimes quite remarkable. For example, six males of the same litter, 13 weeks old, showed counts of 1.87, 1.84, 1.89, 1.87, 1.84, and 1.94 million nucleated cells per cubic millimeter, respectively; three males and three females from one litter just over 8 weeks of age showed 2.36, 2.01, 2.19, 2.41, 2.32, and 2.02 million nucleated cells, respectively; and in 2 males and 2 females from a litter 6 weeks old, 1.93, 2.12, 2.41, and 2.30 million, respectively. The last two of these groups illustrate the similarity in values obtained for both sexes of the same age and litter. Among 32 groups consisting of from 2 to 8 rats each, the average difference between litter mates of the same age was 281,000 cells per cubic millimeter. This approximates the accuracy obtained in counting the red blood cells in the peripheral blood.

The variations observed in certain pathological conditions are reserved pending the completion of more extensive studies. It may, however, be mentioned that in inanition of varying degrees there occurs a rapid and large decrease in the number of nucleated cells which is confirmed by histological examination of bone marrow sections.

SUMMARY

Examination of the bone marrow of the femur in the albino rat by a modification of the methods used for counting blood cells gives results which are reproducible within a range of 2 to 3 thousand nucleated cells per cubic millimeter in litter-mate rats of the same age. In normal rats there is an increase in the number of nucleated cells in the bone marrow during approximately the first 3 weeks of life, after which the count remains constant or falls slightly.

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THE BLOOD CLEARANCE AND RENAL EXCRETION OF BILE ACIDS FOLLOWING THE INTRAVENOUS INJECTION OF CHOLIC AND DESOXYCHOLIC ACIDS[1,2]

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The metabolism of the bile acids has been studied by the feeding or intravenous injection of whole bile or of conjugated cholic acid. The injected bile acid was found to leave the blood and appear in the bile within two hours (1). A small fraction escaped in the urine (1, 2).

New methods for the estimation of all types of bile acids, especially desoxycholic acid, were required, in order to obtain further information on the metabolism of bile acids. The author devised a procedure, based in principle upon the hemolytic properties of the bile acids (3). This method estimates the aggregate hemolytic effects of the individual bile acids in the body fluids and expresses them in terms of desoxycholic acid, the most actively hemolytic of the tested bile acids. The probable presence of this bile acid in the blood (4) and the possible physiological and clinical significance of increases of this bile acid in the body fluids has already been suggested (3).

The blood clearance and renal elimination of bile acids following the injection of desoxycholic acid was compared with that of cholic acid to determine essential differences in physiological or metabolic behaviour between these principal types of bile acids occurring in man. Certain biological differences have already been noted (5).

EXPERIMENTAL. The sodium salts of desoxycholic acid (Riedel-de Haen) and cholic acid were injected rapidly into the saphenous or ear veins of normal fasting dogs. The doses used are stated in terms of the acids. Concentrations of desoxycholate as high as 20 per cent and of cholate as high as 16 per cent were used dissolved in physiological saline or in 0.1 molar phosphate buffer solution, pH 7.0. The desoxycholate solution in buffer required readjustment to pH 7.0. The results obtained were the same whether saline or buffer solutions were used.

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Blood samples were collected by arterial puncture. Urine was collected quantitatively by catheter. Water was fed by tube, 90 minutes before injection to assure standard fluid intake and ample urinary output. The bladder was emptied immediately before injection. Mature dogs weighing 12 to 24 kilos were used. The doses were well tolerated aside from prompt hemoglobinuria following the injection of larger doses. Body weight was usually maintained days after the injection. In most instances fresh dogs were used for each experiment. Anesthesia was not required.

Samples of blood and urine were collected 20, 60, 120, 180, 240 minutes after injection. Nine cubic centimeters of urine and whole oxalated blood were examined in duplicate. Results were read by the artificial light of a 150 Watt daylight electric bulb transmitted through glazed glass, the test tubes being held directly against the glass surface. A special sectional test tube rack was devised for this purpose (6). The blood and urine of normal dogs were usually found to contain one milligram per cent or less, if any, of desoxycholate hemolytic equivalents. In several instances the bile acid content of the blood reached 2.0 mgm. per cent, that in the urine 3.5 mgm. per cent, figures approximating those in man (3).

Rate of elimination of bile acids from the blood. Doses representing 10, 20, 40, 60, 80, and 100 mgm. of desoxycholic acid were injected intravenously. The injection was completed within a minute. The findings in the blood at timed intervals are recorded in table 1. A definite elevation in the blood concentration was first noted when 20 mgm. of desoxycholic acid per kilo were injected. With larger doses, a maximum blood concentration of six milligrams per cent desoxycholate hemolytic equivalents was reached. The first determination, made 20 minutes after the injection, indicated an elevated concentration. Determinations made at 60 minutes after injection indicated a slight rise in bile acid content of the blood above the 20 minute level in some instances. At 120 minutes, the concentration fell but was still raised above the control level. The normal blood level was reached in 180 minutes. In some instances, the blood concentration again rose slightly at the end of 240 minutes after injection (expts. 3, 6, 12).

The blood is cleared in less than 20 minutes following the injection of restricted doses of cholic acid. When, however, as much as 300 mgm. of cholic acid per kilo was injected, the blood concentration reached six milligrams per cent of desoxycholate hemolytic equivalents. The blood rise continued for 180 minutes (expt. 11).

Urinary excretion following the injection of bile acids. The bile acid concentration of the urine at time intervals corresponding with those in the blood is recorded in table 1. Doses of 20 and 40 mgm. desoxycholic acid which caused definite rises in blood concentration caused practically no change in urinary concentration. The reverse is true following the injection of cholic acid. A markedly increased bile acid excretion reaching a

TABLE 1

Blood clearance and urinary excretion of bile acids following the intravenous injection of desoxycholic and cholic acids

EXPERIMENT NUMBER	BILE ACID	INTERVALS AFTER INJECTION											
		Control		20 minutes		60 minutes		120 minutes		180 minutes		240 minutes	
		Blood	Urine	Blood	Urine	Blood	Urine	Blood	Urine	Blood	Urine	Blood	Urine
	mgm. per kilo	*mgm. per 100 cc.**		*mgm. per 100 cc.*		*mgm. per 100 cc.*		*mgm. per 100 cc.*		*mgm. per 100 cc.*		*mgm. per 100 cc.*	
	Desoxycholic acid												
1	10	0.0	3.5	0.5	q.n.s.	1.0	q.n.s.	0.5	1.0	0.5	1.0	0.0	2.5
2	20	0.0	3.5	2.5	2.0	3.5	0.0	2.5	1.0	1.0	1.0	2.0	3.0
3	40	0.0	0.5	5.0	2.0	6.0	2.0	3.0	1.0	1.5	1.0	3.5	0.0
4	60	0.0	0.0	3.5	3.0	5.5	2.5	2.0	0.0	0.0	0.0	1.0	0.0
5	80	2.0	2.0	3.5	20.0	3.5	10.0	2.5	0.5	2.5	q.n.s.	1.0	q.n.s.
6	100	0.0	0.6	3.5	3.5	6.0	2.0	2.0	1.0	0.0	0.0	2.5	0.0
	Cholic acid												
7	40	0.5	0.5	0.5	1.0	1.0	2.5	0.5	1.0				
8	60	0.5	0.5	2.0	8.0	2.0	2.5	0.5	0.0	0.5	0.5	0.5	1.0
9	100	0.5	0.0	0.5	5.5	0.5	4.0	0.5	2.0	1.5	0.0	1.0	1.0
10	170	1.0	0.0	0.5	12.0	0.0	7.0	0.0	0.5	1.0	0.5	0.0	1.0
11	300	0.0	0.0	6.0	71.0	5.0	71.0	5.0	75.0	5.0	49.0	2.0	25.0
	Desoxycholic												
12	40	0.5	0.0	3.5	13.0	6.0	0.0	3.5	0.5	0.5	0.0	2.5	5.0
	Cholic												
	170												

* Concentrations, milligrams per 100 cc., expressed as desoxycholate hemolytic equivalents. To translate into cholate hemolytic equivalents multiply by factor of 7.5.

Protocols. Experiment 1. Dog 1, bitch, 12.7 kilos. Four per cent solution of desoxycholate was used. The total amount injected was 130 mgm. The total urine voided in four hours was 42 cc.

Experiment 2. Dog 2, male setter, 18.6 kilos. Two per cent desoxycholate was injected. The total amount injected was 360 mgm. The total amount of urine voided in four hours was 190 cc.

Experiment 3. Dog 3, male collie, 18.1 kilos. A 4 per cent desoxycholate solution was injected. The total amount injected was 720 mgm. A total of 355 cc. of urine was voided in four hours.

Experiment 4. Dog 4, bitch, 17.1 kilos. A 10 per cent desoxycholate solution was injected. The total amount injected was 1020 mgm. The total voided was 127 cc.

Experiment 5. Dog 5, bitch, 12.7 kilos. A 10 per cent desoxycholate solution was

injected. The total amount injected was 1020 mgm. Fifteen cubic centimeters of urine were voided in four hours.

Experiment 6. Dog 6, bitch, 15.3 kilos. A 20 per cent desoxycholate solution was used. The total amount injected was 1530 mgm. A total of 45 cc. of urine was voided.

Experiment 7. Dog 7, female hound, 17.4 kilos. An 8 per cent cholate solution was used. A total of 700 mgm. was injected. A total of 270 cc. of urine was voided in two hours.

Experiment 8. Dog 8, bitch, 17.3 kilos. A 10 per cent cholic acid solution was injected. The total amount used was 1040 mgm. Two hundred seventy cubic centimeters of urine were voided in four hours.

Experiment 9. Dog 9, female hound, 16.5 kilos. A 16 per cent cholate solution was injected. The total amount injected was 1600 mgm. Three hundred forty-five cubic centimeters of urine were voided in four hours. The equivalent of 80 mgm. of cholic acid was excreted in the urine in 60 minutes after the injection.

Experiment 10. Dog 10, male police dog, 23.7 kilos. Sixteen per cent cholate solution was injected. The total amount injected was 4030 mgm. Five hundred and thirty cubic centimeters of urine were voided in four hours. The equivalent of 105 mgm. of cholic acid was excreted in the urine in 60 minutes after the injection.

Experiment 11. Dog 8, bitch, 15.6 kilos. Ten per cent cholic acid was injected. A total of 4700 mgm. was injected. A total of 300 cc. of urine was voided in four hours. Approximately, the equivalent of 1450 mgm. were excreted in the urine in three hours, 800 in the first hour.

Experiment 12. Dog 3, male collie, 18.6 kilos. Four per cent desoxycholate, and 16 per cent cholate solution were injected. The total quantities injected were 720 mgm. of desoxycholate and 3160 mgm. of cholic acid.

maximum of 75 mgm. per cent of desoxycholate hemolytic equivalents, or about 563 cholate equivalents took place when 300 mgm. per kilo of cholate were injected. The maximum urinary concentration reached 12 mgm. desoxycholate hemolytic equivalents or approximately 90 cholate equivalents when 170 mgm. of cholic acid per kilo were injected. This increased excretion continued for an hour after injection while the blood level was as before injection. When 300 mgm. per kilo were injected, the urinary increase lasted for 3 hours. In some experiments, definite diuresis accompanied the urinary rise in bile acid.

The injection of a mixture of desoxycholic and cholic acids produced changes in the blood and urine combining the results obtained with injections of the same doses of the individual bile acids.

The blood-urine bile acid ratio. The normal bile acid content of blood and urine assumes no constant relationship. The blood level may be slightly higher or vice versa. Following injection, however, a definite relationship is established, whenever the blood or urinary concentration is increased above the normal level.

Ratios between abnormal concentrations of bile acids in the blood and urine following the injection of bile acids may be calculated from data in table 1. A quotient is obtained by dividing the concentration of bile

acids in milligrams per cent of desoxycholate hemolytic equivalents in the blood by that in the urine. It is evident that with a single exception (expt. 5), the ratios following the injection of desoxycholic acid give quotients greater than unity (expt. 2, 1.3, 2.5; expt. 3, 2.5, 3.0, 3.0; expt. 4, 1.2, 2.2; expt. 6, 1.0, 3.0). The ratios following the injection of cholic acid all give quotients less than unity (expt. 8, 0.3; expt. 9, 0.1, 0.1; expt. 10, 0.05; expt. 11, 0.1, 0.1, 0.05).

COMMENT. The tested bile acids differ in the rate of clearance from the blood following intravenous injection. When cholic acid was injected, at twenty minutes and thereafter, the blood concentration of bile acids was as before the injection. A rise in the blood concentration first became manifest when 300 mgm. per kilo were injected. The injection of desoxycholic acid, on the other hand, was associated with a rise in the concentration of bile acids in the blood when 20 mgm. per kilo were injected. This increase was demonstrable for 2 hours after the injection. In some instances, the blood concentration was higher at 60 minutes than at 20 minutes. In some instances, a slight rise occurred at 240 minutes after the control level had been reached at 180 minutes. It cannot be stated at present whether these secondary rises depend on the injected bile acid intrinsically or upon bile acids derived from the liver.

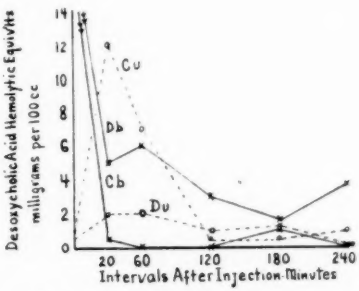

Fig. 1. Bile acid concentration of blood and urine at intervals after the rapid intravenous injection of desoxycholic and cholic acids.

Cu, ○--------○ Urine: 170 mgm. cholic acid injected; *Cb*, x————x Blood: 170 mgm. cholic acid injected; *Du*, ○--------○ Urine: 40 mgm. desoxycholic acid injected. *Db*, x————x Blood; 40 mgm. desoxycholic acid injected.

The reactions to injection of the two acids also differ as to the amounts excreted in the urine. Though the first evidence of bile acid escape in the urine follows the same doses of both acids, cholic acid was eliminated three to twenty times more rapidly by the kidney than desoxycholic acid.

Significant relationships between blood level and urinary concentration may exist when either is elevated above normal. The blood may be elevated, and the urine normal. This occurred when 20 and 40 mgm. of desoxycholic acid per kilo were injected. The urinary concentration may be high, and the blood level normal. This occurred when amounts as high as 170 mgm. of cholic acid per kilo were injected. The blood and urinary concentrations may both be increased. This took place when doses of desoxycholic acid of 60 mgm. per kilo or more were injected. Under these conditions, with a single exception (expt. 5), the bile acid level of the

blood in desoxycholate hemolytic equivalents was higher than that in the urine. The observation is better noted 60 and 120 minutes after injection, than at 20 minutes when the distinction is less sharp.

The type of bile acid retained in the blood following the injection of desoxycholic acid cannot be positively identified as desoxycholic acid at present. It is probably not cholic acid since when cholic acid is injected directly, it promptly leaves the blood. The bile acid eliminated in the urine following the injection of cholic acid may be identified with cholic acid since studies have been reported, using colorimetric methods, which estimate cholic and glycocholic acids and not desoxycholic acid, which indicate a rapidly falling blood concentration and a rise in urinary concentration, at intervals beginning from the time of injection of glyco- and taurocholic acids (1).

Several hypotheses are advanced to account for the differences between the blood clearance rates and the urinary excretion of bile acids following the injection of the two bile acids. They cannot be attributed to renal injury alone since all bile acids probably produce some form of renal injury. Even the synthetic, relatively, non-toxic dehydrocholic acid, is nephrotoxic (7). The fact that when large doses of both bile acids are injected, they are followed by a continued renal excretion in as high a concentration as when they are injected individually, indicates that the retention in the blood cannot be attributed to greater renal injury by desoxycholic acid. Greater adsorption of desoxycholic acid to serum proteins cannot be accepted in explanation since desoxycholic acid has not been found to be bound to serum protein fractions in a greater degree, judging from the inhibitory effect of serum proteins on bile salt hemolysis (8). There may be a renal threshold for the excretion of desoxycholic acid and none for cholic acid. The singular capacity of desoxycholic acid to form addition or coördination compounds with various substances (9) may also possibly explain its delayed removal from the blood.

The major amounts of the injected bile acids are excreted in the bile. The amounts of bile acid lost in the urine following the injection of cholic acid is a small fraction of the entire dose; approximately 2.5 to 4 per cent computed on the basis that cholic acid is the bile acid excreted in the urine. This fraction may reach 30 per cent of the dose when 300 mgm. per kilo are injected.

When the bile acids are injected in restricted dosage, there is a tendency for bile acid retention in the blood following the injection of desoxycholic acid and for the excretion of bile acids in the urine following the injection of cholic acid (fig. 1). The bile acids occurring naturally in the blood and urine may differ fundamentally in type.

Experiments are in progress to determine whether under experimental and clinical conditions, liver injury and biliary stasis may produce sig-

nificant and characteristic changes in blood clearance or urinary excretion of bile acids.

SUMMARY

The blood clearance rates differ following the injection of desoxycholic and cholic acids. The blood is promptly cleared of cholic acid whereas the bile acid concentration of the blood remains elevated for 120 minutes after the injection of desoxycholic acid in restricted doses.

The urinary excretion of bile acid following the injection of cholic acid is several to many times greater than that following the injection of desoxycholic acid. The total amount of bile acid lost in the urine is usually a relatively small fraction of the injected dose in both instances.

With restricted doses of desoxycholic acid, bile acid may not be excreted in the urine, while the blood concentration may rise.

The blood urine bile acid concentration ratio, when the concentration in either fluid is increased above normal, following injection, may assume a specific and constant relationship depending on whether desoxycholic or cholic acid is injected.

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VAGINAL AND UTERINE GRAFTS IN THE RAT AS INDICATORS OF THE PRODUCTION OF OESTRIN

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Recent investigation (Pfeiffer, 1936a) indicates a non-cyclic action of the male hypophysis in the rat and therefore a constant production of the sex hormones. Ovarian grafts in the male, whether normal or castrated, have good follicular development (Goodman, 1934) but luteinization does not occur unless gonadotropic hormones are injected. These same conditions are true of the ovaries of females with masculinized hypophyses (Pfeiffer, 1936a). However, in the latter animals it is much easier to analyze the hormonal state of both the hypophysis and the ovary. The masculinized hypophysis is not able to release luteinizing hormone in sufficient amount to cause luteinization but the injection of it produces all the cyclic action of the ovary (Witschi and Pfeiffer, 1935). There is a balance between the oestrin produced by the ovary and the follicle-stimulating hormone of the hypophysis which results in a non-cyclic and constant production of oestrin that is above the requirement of the vagina for oestrus but below that necessary for full oestrus of the uterus. Since Martins (1932) concluded that cycles were present in the vaginal grafts in castrated males carrying ovarian grafts, it is necessary to investigate more fully the production of oestrin by an ovarian graft in the male. The only adequate method of determining the cyclic production of oestrin in the rat is by the cell proliferation of the vaginal epithelium. However, since the uterus requires a higher level of oestrin, it would appear to be advisable to graft both vaginal epithelium and uterine endometrium into the various animals to be tested.

METHODS. Albino rats from a single commercial strain were used in all of these experiments. The ovary was placed in the right eye after the technique of Goodman (1934), the uterine endometrium and vaginal epithelium in the left eye after the technique of Markee (1929), and the vaginal grafts under the skin after the technique of Martins (1932). Host and donor were 85 days of age. All operations were performed under deep ether anesthesia. The grafts were examined daily under a binocular

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dissecting microscope (×20); but it was necessary to prepare histological sections in order to study in detail the effects on the vaginal epithelium. All grafts, gonads, and accessory sexual organs were removed at autopsy (after 45 days), sectioned at 10μ, and stained with eosin and Delafield's hematoxylin.

RESULTS. *1. Uterine and vaginal grafts in normal females.* Seven females received uterine endometrium and seven received vaginal epithelium in the anterior chamber of the eye, in order to determine the re-

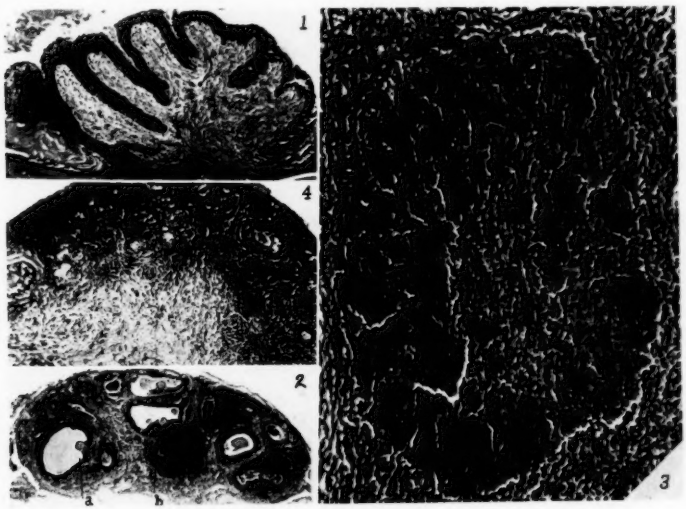

Fig. 1. Photomicrograph of a cross section of the vaginal epithelium graft in the male castrate rat showing the keratinized epithelium. ×45.

Fig. 2. Cross section of an ovarian graft in the castrate male. a. Normal graafian follicle. b. Corpus luteum. ×15.

Fig. 3. The corpus luteum of figure 2 at a higher magnification. Note the characteristic lutein cells. ×75.

Fig. 4. Cross section through an ovarian graft in the non-castrate male showing several primary, but no maturing, follicles. ×45.

sponse to the oestrin produced by the normal ovary. The grafts (homotransplants) remained vitalized in all but one animal in each group. There was a distinct hyperemia of the uterine endometrial graft corresponding to the full oestral change in the intact uterus, but the characteristic alternate blanching and blushing of the endometrium in other species (Markee, 1929, 1931, 1932a and 1932b) was not observed here. Histological sections of the vaginal epithelial grafts made during the various stages of the normal cycle demonstrated the same changes in the graft

as in the intact vagina. Since the uterine endometrial and vaginal epithelial grafts responded to oestrin produced by the ovary in the same manner as if they were in their respective intact positions, they could be safely used as a measure of oestrin production.

2. Vaginal epithelial grafts in males bearing ovarian grafts. Seven normal and six castrated males received $\frac{1}{3}$ of an ovary in the anterior chamber of the left eye and a small piece of vaginal epithelium in the right. As in the case of the normal females (section 1) nothing could be ascertained as to the cell proliferation in the intact vaginal graft. It was found upon sectioning (after 45 days) that the vaginal epithelium was thick and keratinized in the castrates (fig. 1), while in the non-castrates the epithelium was only a few layers in thickness and resembled the dioestrous stage of the intact epithelium.

Histological study of the ovarian grafts from the castrate group showed that numerous follicles were to be found in all stages of development up to the preovulatory stage (fig. 2). In two grafts several degenerating corpora lutea were found, one of which still showed the fairly typical staining reactions of the normally functioning corpus luteum (fig. 3). In the non-castrates the ovarian grafts contained only a few primary follicles (fig. 4) and in one case only a single follicle. The male accessory sexual organs demonstrated a suppression of the male hormone in these non-castrates as previously described by Pfeiffer (1936b).

The absence of keratinization of the vaginal grafts in the non-castrated males may be explained by the condition of the ovarian grafts which obviously produced very little oestrin. However, in the castrate, where the ovary was in equilibrium with the male hypophysis, the oestrin level was high enough to cause keratinization of the vaginal epithelium. This indicated a full oestral condition as far as the vagina was concerned. There was no reaction that could be interpreted as pointing toward a cyclic production of oestrin. Therefore, in order to test whether there was a cyclic reaction of the vaginal graft in the male, smears were followed on a segment of vagina placed under the skin.

3. Males with vaginal grafts under the skin. Twelve male rats received vaginal grafts under the skin according to the technique of Martins (1932). All received ovarian grafts and six were castrated at the same operation. The castrated group received a 21 day old ovary in the right eye and the normal group received an ovary in the right testis.

In the normal group the vaginal grafts did not all survive. It was thought unwise to inject oestrin (as suggested by Martins, 1932) to establish the graft as that would suppress the hypophysis and indirectly injure the ovarian graft. Neither was it desirable to inject gonadotropic substances as they would, at least for a time, influence the reaction of the ovary and cover up the true action of the ovarian graft. Only three vagi-

nal grafts proved successful enough so that daily smears could be made. In these animals there was continuous musification. It had been shown by Myer and Allen (1931 and 1932) that musification could be produced by the action of less than one half the oestrin required for cornification.

In the castrate group five of the vaginal grafts were sufficiently successful to allow daily smears to be followed for at least a month. In all of these there was a continuous oestrus except for a few brief, rather irregular, interruptions. The cornified cell stage lasted from 15 to 25 days and was interrupted only by a short dioestral smear or leucocytic stage of about 36 hours. This was not interpreted as a cyclical phenomenon.

The histological appearance of the ovarian grafts was similar to that found in the group in which the vaginal epithelium was placed in the eye. As in the other experiments the difference in the reaction of the vaginal epithelium in the castrate and non-castrate groups was undoubtedly due to the physiological condition of the ovarian graft.

4. Uterine endometrial grafts in males bearing ovarian grafts. Six normal and six castrated males received ovaries in the left eye. At the same operation a small piece of uterine endometrium was placed in the right eye. Two of the non-castrate animals were autopsied at 25 and 35 days respectively because of a respiratory infection, the so-called "snuffles." All others were autopsied after 45 days.

Daily observations failed to show any hyperemic condition such as found in the female at oestrus. On histological section the endometrium resembled that found during dioestrus in the intact position. There was no difference in reaction between the castrate and the non-castrate groups except that the animals with "snuffles" infection showed very degenerative grafts. The ovarian grafts were the same as those in the previous experiment except one case, in the non-castrate group, which contained many developing follicles. As this single host showed no deficiency of the male hormone, a common factor was indicated as the cause of suppression of both the testis and ovarian graft. It was clear, however, that the amount of oestrin released by the ovary in the male, whether castrated or normal, was below that required for the oestrus of the uterus.

Discussion. The above experiments demonstrate that the ovarian graft in the male releases oestrin in a non-cyclical manner and at a fairly constant level. However, the fact that there is no alternate blanching and blushing of the uterine endometrium in the eye of the rat is in marked contrast to that observed by Markee (1929, 1931, 1932a and 1932b) for the rabbit, guinea pig and monkey. There are two possible explanations for the absence of this phenomenon: 1, it may be due to a pressure factor, as the anterior chamber of the eye is small, or 2, there may be a species difference. There is some evidence (unpublished work) that both are contributing factors. An explanation of the absence of the alternate

blanching and blushing is not essential to the interpretation of the results of these experiments. There is a characteristic hyperemia of the endometrial graft during the oestrus of the normal female which never occurs in the male. This and the constant cornification of the vaginal epithelium demonstrate that the oestrin level, as in the females with masculinized hypophyses, is above that required for the vaginal cornification but below that necessary for oestrus in the uterus. The absolute parallelism between the physiological action of oestrin in the female with the masculinized hypophysis and that produced by the ovarian graft in the male, indicates that the release of hormone from their hypophyses is also similar and consists of only follicle stimulating hormone.

The results of daily smears of the vaginal grafts under the skin are essentially the same as those of Martins (1932). He observed cornified cells for 6 to 15 days, leucocytes for 1 to 2 days, and then another long oestrous phase. The longer cornified cell stage in our investigation, due to the eye being a better transplantation site for the ovary, demonstrates that it is really a state of continuous oestrus. The break in the continuous oestrus is due to transplantation factors (Pfeiffer, 1934) and is not an indication of a cyclic action of the ovarian graft or the hypophysis of the male.

Since there is no evidence of cyclic release of oestrin, it is of interest that corpora lutea are sometimes present 45 days after transplantation of the mature ovary into castrate males. It is likely that they have persisted from the time of transplantation as Smith (1930) has shown that corpora lutea may persist for a long time in the ovary of the hypophysectomized female. However, Pfeiffer (1936a) has definitely shown that unless the testis has been present in the pre-pubertal stage, corpora lutea regularly form in the grafted ovary in the male. Therefore, the time of castration is important and in this case, as well as the few that have been reported (Moore, 1919, and Wang, Richter and Guttmacher, 1925) the corpora lutea may have formed after transplantation as Evans et al. (1935) have shown that some luteinizing hormone is present in the hypophysis of the castrate male. The male hypophysis does not normally release luteinizing hormone at least in amounts sufficient for luteinization, as the ovarian graft in the male (normal or castrate) shows only follicular development.

The observations that the ovarian grafts are usually degenerative in character and the male accessory sexual organs are suppressed in the non-castrate males may be taken as an indication of mutual antagonism between the testis and ovary. However, present investigation (in progress) suggests that it may be due to the toxic factors introduced by the so called "snuffles" infection. The demonstration by Myers and Allen (1931, 1932) that musification of the vagina is produced by suboestral amounts of oestrin explains the musification observed in the non-castrate group where the ovarian grafts were suppressed.

SUMMARY AND CONCLUSIONS

1. Uterine endometrium and vaginal epithelium were successfully transplanted into the anterior eye chamber of the rat. Hyperemia of the uterine endometrial graft during oestrus enabled the cycles of the normal female to be followed by this method. However, cycles could be demonstrated in the grafted vaginal epithelium only upon histological examination.

2. In male rats, either normal or castrated, the uterine endometrial grafts gave no evidence of complete oestral reaction to the oestrin produced by an ovarian graft. The vaginal epithelium was continually cornified in the castrate group but in the non-castrate group the suppressed ovaries were not able to produce more than enough oestrin to cause musification.

3. The uterine endometrial and vaginal epithelial grafts demonstrated cyclic function of the ovary in the normal female but not of the ovarian graft in the male. This lends further evidence that the hormone produced or released from the male hypophysis is non-cyclic and that the female hypophysis is essential for the cyclic function of the ovary.

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THE BIOASSAY OF ADRENAL CORTICAL EXTRACTS

A Direct Comparison of Rat and Dog Units

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Harrop, Pfiffner, Weinstein and Swingle (1) and Pfiffner, Swingle and Vars (2) have elaborated a dog method of assay and have defined the dog unit as the minimum daily per kilogram dose of adrenal cortical hormone necessary to maintain normal physiological conditions in the adrenalectomized dog for a period of 7 to 10 days, the two criteria of normal physiologic conditions being maintenance of body weight and blood level of nonprotein nitrogen (or urea). This method has been used extensively by research workers in this field and is generally regarded as the best available method for the bioassay of adrenal cortical extracts.

Adrenalectomized rats have also been used. The fact that rats rarely survive adrenalectomy if the glands are carefully and completely removed was demonstrated by Pencharz, Olmstead and Gerogossintz (3) and Freed, Brownfield and Evans (4). Kutz (5) has studied the use of adrenalectomized rats and has defined the rat unit as the minimum daily dose of cortical hormone which will protect, for at least 20 days, 50 per cent of a group of animals adrenalectomized at 28 days of age, the extract being administered subcutaneously twice daily.

The suitability of young adrenalectomized rats for assaying adrenal cortex extracts has been considered further by Firor and Grollman (6), Gaunt (7, 8), Schultzer (9) and Cleghorn and co-workers (10). A rat method of assay was used by Grollman and Firor (11) for evaluating adrenal cortical extracts.

A review of the literature indicates that there is a great difference of opinion regarding the suitability of adrenalectomized rats for testing adrenal cortical extracts. Most of the disagreement seems to center in the number of indefinite survivals encountered among untreated controls and after cessation of treatment with hormone. If the adrenalectomized rat is to be regarded as a satisfactory animal for assay of adrenal cortical extracts, there should be no large incidence of indefinite survivals among controls, and the rats successfully maintained by hormone therapy should die in the normal time after cessation of injections.

The rat method is less time-consuming than the dog method, which constitutes a decided advantage in following the hormone distribution in connection with fractionation studies. However, we have noted no reports in the literature where the rat and dog assays have been run in parallel, and it appears that this is necessary before any high degree of confidence can be placed in the rat method. The present study is concerned with a direct parallel comparison of the rat and dog methods applied to the same adrenal cortical extracts for the purpose of establishing the relative values of the dog and rat units respectively.

EXPERIMENTAL. The adrenal cortical extracts used in these studies were prepared by a method developed in this laboratory, which will be described in a separate report. The extracts were made up so that 40 grams of fresh beef adrenal glands were represented in each cubic centimeter. These extracts contained from 0.6 to 1.0 mgm. of gland extractives per cubic centimeter and were substantially free of epinephrin and other toxic impurities. The solutions assayed by the dog blood pressure method showed less than 1 part of epinephrin per 400,000.

The dog method. This method has been followed throughout as described by Pfiffner, Swingle and Vars (2), except that the total non-protein nitrogen increase was followed, instead of urea nitrogen. Non-protein nitrogen was determined by the method of Koch and McMeekin (12) on the trichloracetic acid filtrate of the blood. The technique for removal of adrenals from dogs, described by Banting and Gairns (13) was followed.

The rat method. Young male rats of Wistar strain, 4 weeks old and weighing 50 to 60 grams, were used. The rat rooms were kept at 75 to 80°F. The diet consisted of Purina Dog Chow upon which the rats show excellent growth without need of dietary supplements. The analysis given by the manufacturers is sodium 0.67 per cent, chloride 0.68 per cent.

The rats were anesthetized with sodium amytal 60 mgm. per kilo intraperitoneally which was supplemented with a little ether when necessary. The operative field was shaved and washed with 70 per cent alcohol. An oblique incision was made on the right side in the lumbar region, and the muscles divided parallel and posterior to the lowermost rib. The right kidney can be easily pushed forward through the incision. By gently tilting up the anterior pole of the kidney, the adrenal gland with its pedicle was clearly exposed. The pedicle of the adrenal was grasped anteriorly of the gland by means of forceps and the pedicle cut with scissors just anterior to the forceps. The gland and the fatty tissue in which it is embedded were then lifted upward and backward. In this way one-third to one-half of the capsule of the kidney is removed which insures a clean removal of all fatty tissue around the adrenal gland. By grasping the cut edge of the muscle and gently lifting it, the kidney can easily be made to

drop back into its normal position. The muscle and skin were brought together with a single silk suture. The same procedure was used for removal of the left gland except that the incision was made about 1 cm. more posteriorly and just dorsal to the upper part of the spleen. Each rat was wrapped in cotton from which he could extricate himself when no longer anesthetized. By means of this technique, forty to fifty can easily be adrenalectomized in six to seven hours by one operator. The instruments and silk sutures are kept in 70 per cent alcohol and the operator's hands washed with this solution. On the third or fourth day, when the wounds are already tightly closed, the sutures are removed.

The hormone solutions were administered by single subcutaneous daily injections beginning on the day of operation and continued for 20 days. Not less than 5 uninjected controls were used with every series, the usual

TABLE 1

Survival period after adrenalectomy in rats

GROUP	NUMBER OF RATS OPERATED	AVERAGE SURVIVAL AFTER OPERATION	NUMBER SHOWING INDEFINITE SURVIVAL
		days	
I. Controls not injected	192	6.88	5
II. Injected for 20 days with active extracts	240	27.21*	8
III. Injected with extracts which were practically inactive	157	7.27	4
IV. Injected with extracts of low activity	86	16.24	2
Total	675		19

* These rats survived on an average 7.21 days after discontinuing hormone injections.

procedure being to inject parallel groups of 5 rats each with 0.1, 0.2, and 0.3 cc. respectively of hormone solution.

RESULTS. Up to the present time 675 rats have been adrenalectomized by the above described technique. This number comprises four groups and the data concerning their survivals are given in table 1. In the control group of 192 rats the average survival after operation was 6.88 days. In group II, the 240 rats which received maintenance dosages of extract for 20 days showed an average survival of 7.21 days after discontinuing injections. In group III, the 157 rats which were injected with inactive extracts showed an average survival of 7.27 days after operation which is essentially the same as that of the control group. The 86 rats in group IV received sufficient doses of extract to definitely increase their survival period over that of controls but not sufficient to maintain them for 20 days, the routine period of injection.

Whenever an adrenalectomized rat survived more than 15 days after operation or after cessation of injections, it was considered as an "indefinite survival" and search was made on autopsy for residual adrenal cortex tissue. Such animals must be excluded in calculating rat units, although they have been included in table 1. Of the total 675 rats listed in table 1, only 19 showed indefinite survival, and in 14 of these, adrenal rests were found on autopsy to account for the prolonged survival. Thus, in our experience the mortality in rats after adrenalectomy is 97 per cent and

TABLE 2

Adrenal cortical extracts assayed in rat units

EXTRACT NUMBER	DAILY DOSE (1 CC. REPRESENTS 40 GRAMS GLAND)	NUMBER OF RATS INJECTED	NUMBER OF RATS SURVIVING ON 20TH DAY	AVERAGE GROWTH IN 20 DAYS	AVERAGE SURVIVAL AFTER LAST INJECTION	RAT UNITS PER CUBIC CENTIMETER OF EXTRACT	
	cc.			*grams*	*days*		
245 MHK-2	0.3	5	5	23	5		
	0.2	5	5	24	7		
	0.15	5	5	30	8		
	0.10	5	4	15	6.5	>6.6	8.3
	0.05	6	4	8.5	4.5	<10	
292 MHK-2	0.3	5	5	22	4.0	>3.3	4.1
	0.2	6	5	13	5.4	<5.0	
	0.1	6	0				
185 MHK-2	0.3	5	5	37	9.3		
	0.2	5	5	20	5.2		5.0
51 MHK-5	0.3	5	4	18	4.0		
	0.2	5	5	21	5.4		5.0
	0.1	5	0	5			
230 MHK-2	0.3	5	4	24	6.0	>3.3	4.1
	0.2	5	4	12	4.3	<5.0	
11 MHK-5	0.3	7	6	25	5.0	>3.3	4.1
	0.2	6	4	20	4.5	<5.0	

the average survival of those rats which are adequately maintained for 20 days by daily injections of hormone is practically the same after cessation of injections as the average survival period of the uninjected controls.

The next step was to define a rat unit in such a way as to permit a quantitative estimation of the hormone content of various adrenal cortical extracts. After a series of preliminary experiments, the rat unit was defined as the minimum daily dose of hormone which, administered by single subcutaneous injection daily for 20 days to 4 weeks old male rats

weighing 50 to 60 grams, is sufficient to protect at least 80 per cent of the rats and produce an average growth of at least 20 grams per rat for the 20 day period. The survival and growth data together with the calculated rat units are given in table 2 for six active extracts assayed by the rat method.

The results in table 2 show that at the higher dosages of extract the growth is not proportional to dosage, which is in agreement with the criticism of the rat method given by Cleghorn and co-workers (10) in a recent report. Consequently, growth alone is not a satisfactory basis of assay. However, it is apparent from the data in table 2 that there is for each extract a certain minimum dose at which not less than 80 per cent of the rats survive and the average growth for the 20 day injection period is not less than 20 grams per rat. This has been selected as the basis of the rat unit. This dose for extract 245 MHK-2 is greater than 0.1 cc. and less than 0.15 cc.; for 292 MHK-2 it is greater than 0.2 and less than 0.3 cc. per rat daily. For 185 MHK-2 the average growth on 0.2 cc. was 20 grams, consequently this was taken as the unit dose. If the dosage is lowered below the minimum which will maintain 80 per cent and bring about an average growth of 20 grams in 20 days, the rate of growth falls off rapidly.

A unit could be based solely upon survival of at least 80 per cent of the rats, and such a unit would be approximately 25 per cent smaller than the one based on survival plus growth. However, we have chosen the larger unit because it more nearly approximates a physiological maintenance unit for the adrenalectomized rat. For convenience we have adopted a single daily subcutaneous injection, although we realize that a divided dosage may give a more effective utilization of the hormone. Also we have some preliminary experiments which indicate that when the hormone is administered in the drinking water the growth may be more proportional to dosage.

Comparison of rat and dog methods of assay. As a check on the value of the rat method as described above, a number of extracts were assayed in parallel by the rat and dog methods, using the dog method as described by Harrop and co-workers (1). The results obtained with eight preparations are given in table 3. As previously mentioned, all extracts were made up so that each cubic centimeter represented 40 grams of whole beef adrenal glands.

The ratio of dog units to rat units varies from 17.8 to 25.4 with an average of 21.7 for the extracts compared. The results given in table 3 indicate that the quantitative estimation of activity by the rat method follows very closely the results obtained by the dog method, although the rat's requirements are much higher thus giving a larger unit. Preparation 245 MHK-2 which gave the highest rat assay also gave the highest dog assay. Preparation 122 MHK-2 gave the lowest dog assay and also the lowest

TABLE 3

Comparison of rat and dog units

EXTRACT NUMBER	RAT UNITS PER CUBIC CENTIMETER	DOG UNITS PER CUBIC CENTIMETER	RATIO $\frac{\text{DOG UNITS}}{\text{RAT UNITS}}$
102 MHK-2	3.3	Dog 1, >40 <80 60	$\frac{60}{3.3} = 18.2$
136 MHK-2	>3.3 <5.0 4.1	Dog 1, >66 <133 100	$\frac{100}{4.1} = 24.4$
230 MHK-2	>3.3 <5.0 4.1	Dog 1, >68 <136 102	$\frac{102}{4.1} = 24.8$
245 MHK-2	>6.6 <10 8.3	Dog 1, >138 <276 207	
		Dog 4, >108 <216 162	
		Dog 5, >73 <146 110	
		Average 160	$\frac{160}{8.3} = 19.3$
292 MHK-2	>3.3 <5.0 4.1	Dog 4, >56 <112 84	
		Dog 7, >42 <84 63	
		Average 73	$\frac{73}{4.1} = 17.8$
14 MHK-5	>3.3 <5.0 4.1	Dog 4, >116 <232 174	
		Dog 6, >40 <80 60	
		Dog 8, >50 <100 75	
		Average 104	$\frac{104}{4.1} = 25.4$
51 MHK-5	5.0	Dog 4, >62 <124 93	
		Dog 8, >84 <168 126	
		Average 109	$\frac{109}{5.0} = 21.8$
122 MHK-2	<2.0	Dog 1, >15 <30 22.5	

rat assay, although for this extract the lower limit in rat units was not established and consequently the ratio of dog to rat units cannot be given.

It is now well established that the survival period and clinical condition of adrenalectomized rats and dogs can be improved by the addition of certain supplements to the diet, particularly sodium chloride. Consequently in using either the rat or dog method it is very important to control the diet rigidly if quantitative estimations are desired. However, we have not been able to maintain indefinitely either completely adrenalectomized rats or dogs by dietary supplements alone without the administration of adrenal cortical hormone.

It is interesting here to compare the hormone requirements of the adrenalectomized dog and rat when calculated on a per kilogram basis. The seven preparations in table 3 for which ratios of dog to rat units have been determined show an average of 101 dog units per cubic centimeter and 4.7 rat units per cubic centimeter. The dog unit represents the physiological maintenance dose per kilogram of dog. However, the rat unit represents the physiological maintenance dose per rat, which during the 20 day period of injection and growth, weighs on an average approximately 65 grams. When both of these maintenance doses are calculated on a per kilogram basis, the dog is found to require 0.01 cc. and the rat $\frac{1}{4.7} \times \frac{1000}{65} = 3.27$ cc. of extract per kilogram. These doses represent 0.4 and 131 grams respectively of fresh beef adrenal glands. Thus, the young growing rat after adrenalectomy requires per unit of body weight more than 300 times as much adrenal cortical hormone as does the adult adrenalectomized dog.

SUMMARY

A rat method of assay has been used in parallel with the dog method in testing a number of adrenal cortical extracts. The rat unit has been defined as the minimum daily dose of hormone which, administered by single subcutaneous injection daily for 20 days to four weeks old male rats weighing 50 to 60 grams, is sufficient to protect at least 80 per cent of the rats and produce an average growth of at least 20 grams per rat for the 20 day period. The dog unit is the same as that defined by Pfiffner, Swingle and Vars (2).

The rat unit is approximately 22 times as large as the dog unit. On a per kilogram basis the young, growing rat after adrenalectomy requires more than 300 times as much adrenal cortical hormone as does the adult adrenalectomized dog.

Under the experimental conditions described, in a group of 675 rats, a mortality of 97 per cent has been observed in rats following adrenalec-

tomy when no hormone was given or after hormone injections were discontinued. Indefinite survival in the other 3 per cent has been accounted for in most cases by finding residual adrenal cortex tissue at autopsy.

The average survival period observed in a control group of 192 adrenalectomized rats receiving no hormone injections was 6.8 days. In a group of 240 adrenalectomized rats which were maintained by 20 daily injections with cortical extracts, the average survival after cessation of injections was 7.2 days or approximately the same as that of the controls.

The results indicate that the rat method shows satisfactory agreement with the dog method in estimating the hormone content of adrenal cortical extracts.

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THE POTENTIAL ANALYSIS OF A PACEMAKER MECHANISM IN LIMULUS POLYPHEMUS

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The nature and properties of fiber potentials have been determined for the peripheral somatic and autonomic nervous systems in considerable detail (Erlanger, Bishop and Gasser, 1926; Heinbecker, 1929). Many correlations between potential form and bodily function have been established (Heinbecker, Bishop and O'Leary, 1936). Before corresponding correlations can be established for the central nervous system it is necessary that the characteristics of ganglion cell potentials be more specifically recognizable. The difficulty of solving this problem rests primarily on the impossibility of eliminating or identifying axon potentials in records of both axon and cell potentials. Indeed, until such times when potentials can be recorded from single cell bodies with shielded microelectrodes, inferences concerning these cell potentials must be drawn from records made with leads subject to the influence of groups of cells and fibers. Evidence of this latter type has been advanced by Fröhlich (1913), by Adrian and Mathews (1928), by Adrian (1931), by Hartline and Graham (1932) and by Eccles (1935). Their findings suggest that the activity potential of certain ganglion cells acting alone, or as a small associated group, is sustained for a much longer time period than is that of their axons. Bishop (1936) has not been able to assign the long potentials found by Eccles to cell bodies of the superior cervical ganglion of the rabbit. The writer has never seen them in studies on the superior cervical sympathetic ganglion of the turtle. In studies of the mammalian cortex the long potentials generally have been regarded as summation phenomena. It seems highly probable that different types of ganglion cells vary considerably in the nature of their potentials so that further results from investigations of ganglion cells possessing various functions and in various species will have to be available before any generalizations as to their nature are advisable.

Previous studies on the ganglionated median cardiac nerve of Limulus polyphemus (Heinbecker, 1933) indicated that this preparation offered certain advantages as material for the study of ganglion cell potentials. The nerve is completely separable from the heart, permitting its investiga-

tion without the complications of muscle potentials which have so far masked all nerve potentials in investigations of the vertebrate heart. The cardiac ganglionic chain of Limulus possesses relatively few cell types and some of the cells are very large. The different cell types are differently distributed throughout the preparation so that it is possible by sec-

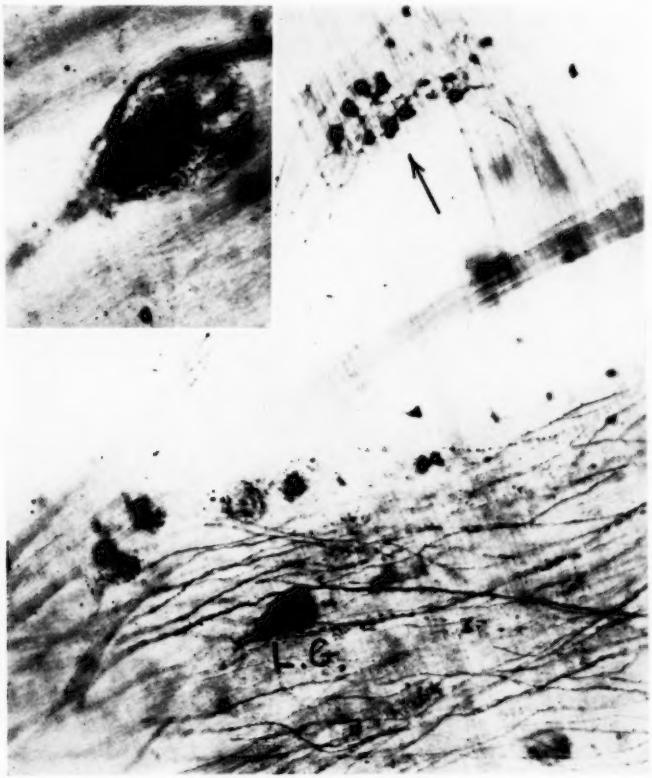

Fig. 1. Photomicrograph of methylene blue preparation of median cardiac nerve of Limulus polyphemus to show large (L.G.) and small (marked with arrow) ganglion cells. Magnification ×66. Insert shows a large unipolar ganglion cell magnification ×220.

tioning to obtain portions of the chain in which some of the types are lacking. Such sectioning of the preparation eliminates spontaneous activity only in the anterior three and the posterior cardiomeres. This is in itself convenient and leads to the inference that cells of the type possessed by these four cardiomeres do not by themselves display the spontaneous

activity shown by certain other cell types. While portions of the ganglionic chain display an intrinsic activity they can also be artificially excited.

The median cardiac nerve contains many non-myelinated fibers of various diameters which in some instances extend nearly throughout the length of the structure. These can be artifically stimulated and their potentials investigated without interference from ganglion cell potentials. In this report there are presented the results of an investigation of the potentials of the median cardiac nerve, particularly as they seem to offer a means of differentiation between fiber and ganglion cell potentials. The results seem to indicate that there is at least one type of ganglion cell which alone or in association with a small number of cells of a second type produces a potential having a duration longer than that of the single axons. This sustained potential of the ganglion cells is associated with repetitive responses of the axons. The effect of certain drugs, such as strychnine, adrenalin and cocaine, on the sustained potential has been the subject of investigation.

For an interpretation and understanding of the result of the potential analyses it is essential to present a brief résumé of certain of the cytological studies of the median cardiac nerve. Reference is made to the work of Patten (1912). It has been our privilege to study much of his original material.[1] We have also made preparations according to the method of Bodian (1936).[2] The ganglion cells of the median cardiac nerve are of several types (fig. 1). There are first giant unipolar cells measuring approximately 140 x 100 microns in methylene blue preparations. In the silver preparations there is considerable shrinkage, the average values for the greatest diameters ranging from 80 to 110 microns. These cells are not found in all cardiomeres, being absent in the first three and the last cardiomeres. Their axons extend in many instances almost the entire length of the cord. All their collaterals appear finally to pass out of the cord into the general nerve plexus of the heart. Scattered among the giant unipolar cells there are a few typical bipolar cells. These are somewhat smaller than the unipolar cells having an average diameter which is less by some 15 to 20 microns. A third type of cell is multipolar and measures from 30 to 60 microns in diameter in the silver preparations. These cells are very numerous and cover the outer portion of the entire nerve cord. They are less numerous in the anterior three or four segments. They have many collaterals which pass out into the general heart plexus.

For the investigation reported in this paper, the nerve cord was studied functionally and histologically as a whole and also in segments. These

[1] Dr. William Patton kindly loaned me some of his Limulus methylene blue preparations.

[2] I am indebted to Mr. R. S. Snider for making the preparations and counting the nerve cell.

segments were cut shorter and shorter until finally a very simple potential form was obtained. The recording apparatus consisted of a three-stage condensor coupled amplifier in association with a cathode ray oscillograph. Early in the work the coupling condensors used were of one-tenth microfarad capacity and the grid leaks one megohm. Because of the duration of the potentials obtained in the investigation the condensors were later changed to ones with two microfarads' capacity. This change permitted the recording of potentials lasting several hundred sigmas with very little

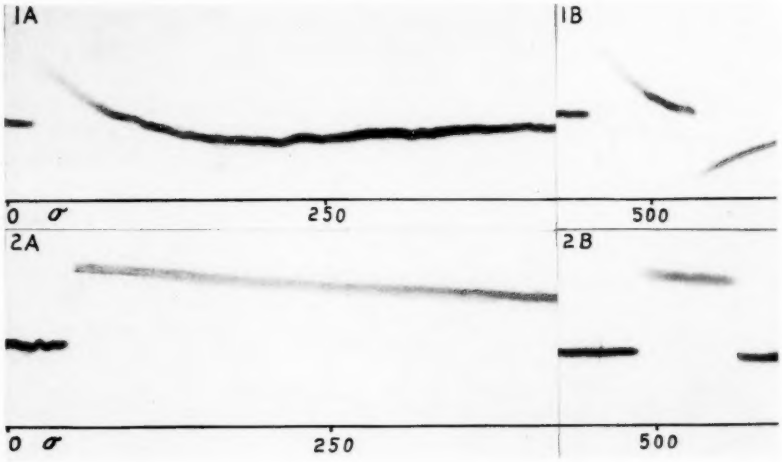

Fig. 2. Calibration of apparatus.

1A, record of output on closure of a constant current key, 0.1 microfarad coupling condensers, 1 megohm leaks, 250,000 amplification.

1B, record of output from input of constant current of relatively short duration, 0.1 microfarad coupling condensers.

2A, record of output on closure of a constant current key, 2 microfarad coupling condensers, 1 megohm leaks.

2B, record of output from input of a constant current of relatively short duration, 2 microfarad coupling condensers.

distortion. A calibration of the system is shown in figure 2. Potentials of the duration under investigation were considerably distorted by the small condensors and tended to show a diphasic artefact simulating a positive after potential. Records of the latter type were used only for certain phases of the investigation in which the actual form and duration of the potentials were not a first consideration. The recording electrodes were of the silver–silver chloride type and were so arranged as to permit a ready alteration of their position with reference to one another and to the preparation.

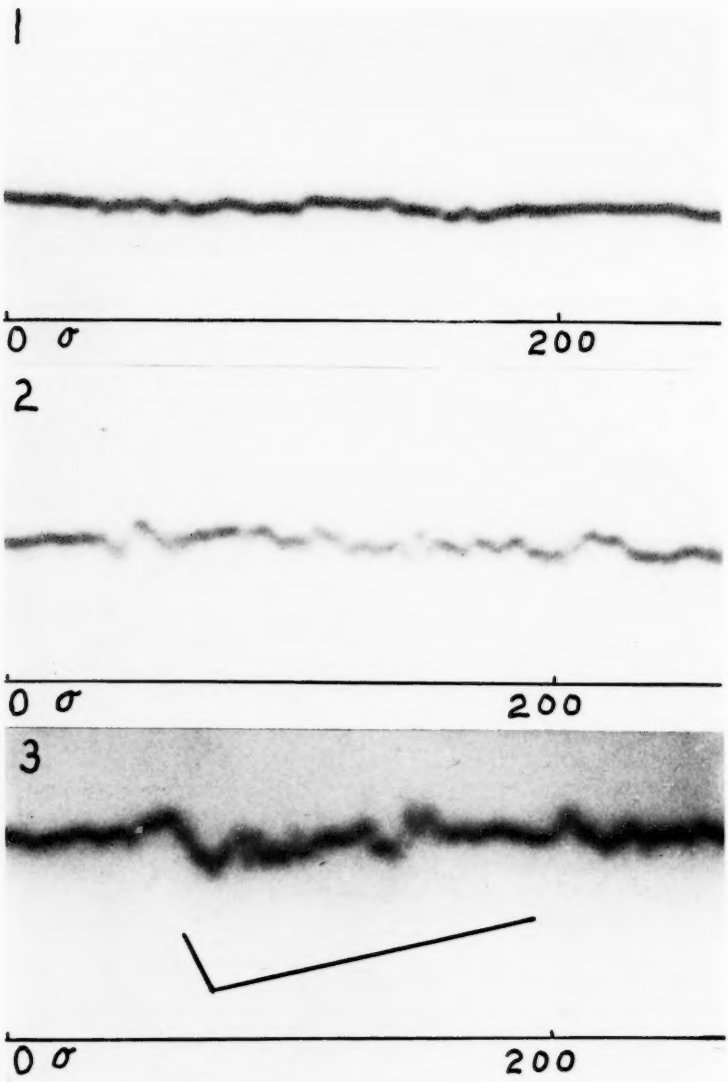

Fig. 3. 1. Electroneurogram from portion of median cardiac nerve corresponding to second cardiomere; whole nerve cord intact because this part of structure is not spontaneously active. Note low potentials with no evidence of a sustained potential.

2. Electroneurogram from portion of median cardiac nerve of the same Limulus corresponding to the 3rd cardiomere; whole nerve cord intact. Note some increase in amplitude of potentials, still no evidence of a sustained potential.

3. Electroneurogram from portion of median cardiac nerve corresponding to the 5th cardiomere; whole nerve cord intact. Note evidence of a sustained potential. In this portion of a nerve cord are found large pacemaker cells. Multipolar cells are present in all portions of the nerve cord.

The complex potential record of the median cardiac nerve. The spontaneous potential record from the median cardiac nerve of Limulus, when recorded with two electrodes on live tissue, consists of periodic outbursts of irregular oscillations with positive and negative phases. These periodic discharges have the frequency of the heart beat. The form of the record varies in some important details in the different sections of the nerve cord. When the recording electrodes are placed on that part of the cord corresponding to the first three cardiomeres, the recorded potentials are lower than when similar leads are taken from the cardiomeres in the midportion of the nerve cord (fig. 3). Furthermore, there is never any evidence of the slow potentials which are nearly always present when the record is from the midportion of the nerve trunk. Very occasionally such slow potentials are not distinguishable in the record even from this portion of the nerve trunk. Even when present the slow potentials are not always visualized throughout their entire length. Their start is nevertheless sufficiently characteristic to always permit their identification (fig. 4).

In the light of evidence to be presented subsequently these findings assume significance. In that part of the nerve cord corresponding to the first three cardiomeres there are regularly none of the large unipolar ganglion cells which might therefore be held responsible for the slow potentials. In the midportion of the nerve cord there are collected all the giant unipolar ganglion cells and it is here that the slow potentials are developed. In those occasional instances where no slow potentials are recognizable in the records made from this portion of the nerve cord it is conceivable that they are developed simultaneously at both electrodes and consequently neutralize each other, only fiber potentials then being recorded.

Potential record from successively shorter stretches of nerve cord. Potentials recorded from two electrodes 10 to 14 mm. apart, each on live nerve from the mid-sections of the nerve cord show a slow potential with short spike potentials superimposed. When the tissue under one electrode is killed the superimposed potentials are thereby rendered more monophasic while the long lasting potential is unchanged (fig. 5). This is the result which would be expected if the brief potentials are conducted fiber potentials. Their complete monophasicity is not to be expected because it has been shown that non-myelinated fiber potentials show a large positive after potential (Bishop, 1934).

Potentials recorded from shorter and shorter stretches of nerve cord after killing under one electrode become simpler and simpler until in favorable preparations a single sustained negative deflection is obtained (figs. 5 and 6). On this single negative potential there are generally superimposed spike potentials of shorter duration and of the type characteristic of nerve fibers. Such spike potentials may be almost absent. The recorded magnitude of the large slow potentials varies with the freshness of

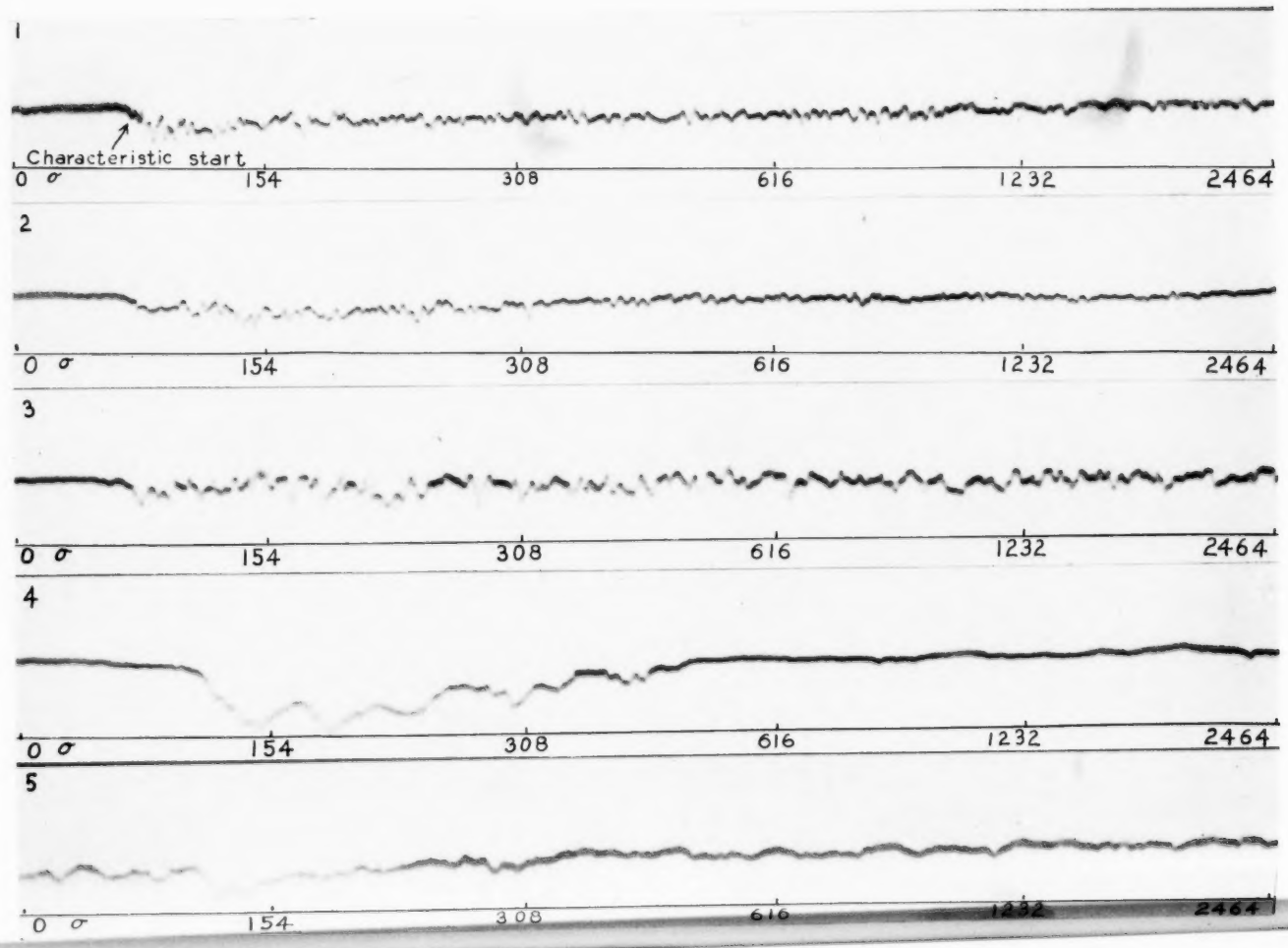
1
Characteristic start
0 σ
154
308
616
1232
2464
2
0 σ
154
308
616
1232
2464
3
0 σ
154
308
616
1232
2464
4
0 σ
154
308
616
1232
2464
5
0 σ
154
308
616
1232
2464

Fig. 4

the preparation from 0.1 to 0.25 millivolt. Their duration varies from 150 to 300 sigmas. Their duration under normal conditions varies with the frequency, being shorter when the rate of their occurrence is rapid. Their rising phase is approximately one-twentieth to one-tenth of the total duration. The duration of the slow potential remains unchanged by shortening the interelectrode distance.

Effect of altering position of the electrode with reference to a point of negative potential. Alteration of the position of an electrode to one or the other side of the nerve cord is without appreciable effect on the potential record. Shifting an electrode longitudinally 3 to 4 mm. with reference to a point of maximum negativity results in a lowering of the recorded potential without any appreciable change in its duration. There may be a reversal of the direction of the potentials when both electrodes are on live nerve. This might be determined by the relation of the cell to the electrodes. When one electrode is kept fixed on killed tissue, moving the other for some millimeters does not change the polarity. These experiments were made in the hope that some evidence might be secured of a directional polarity in the source of potential. The results seem to indicate that the entire cell surface becomes negative to the axon.

Correlation of functional and histological studies. The small portions of nerve cords, 3 to 6 mm. in length, from which smooth sustained potentials had been recorded, were fixed and stained according to the silver-on-slide method of Bodian. In serial sections, 10 to 30 microns in thickness, the nerve cells were identified as to type and counted. In two out of eight preparations a single large unipolar pacemaker cell was found in association with, in one case twenty-three of the multipolar cells, and in another thirty-seven. In the other preparations from two to ten giant unipolar cells were found in association with a somewhat larger number of multipolar cells. The two instances in which such single unipolar cells were found suggest the inference that here the long potential could have been the result of a single unipolar cell acting alone, because in regions where

Fig. 4. 1. Electroneurogram from portion of median cardiac nerve 8 mm. in length, 4 mm. interelectrode distance, rate of spontaneous discharge 24 per minute; 2 microfarad coupling condensers.

2. Electroneurogram after killing under grid electrode. Note marked simplification of record.

3. Electroneurogram 3 minutes after applying 1-2,000 strychnine sulphate. Note increased complexity and duration of record from activation by strychnine, rate 33 per minute.

4. Record after killing further under grid electrode, 3 mm. live nerve left. Rate 30 per minute. Note increased amplitude and duration of sustained potential as compared with 1.

5. Same preparation 4 minutes after application of 1-500 acetylcholine in sea water. Note lowering and shortening of sustained potential, rate 5 per minute.

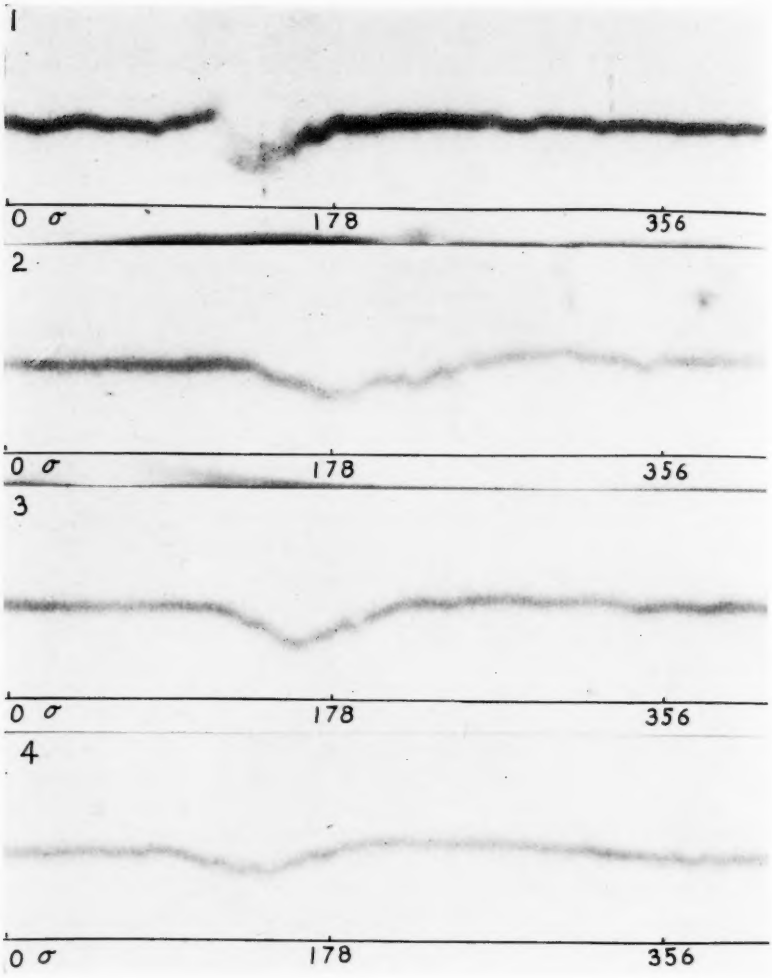

Fig. 5. 1. Electroneurogram from midportion of median cardiac nerve; total nerve length 14 mm., interelectrode distance 12 mm., both electrodes on live nerve. Coupling condensers 0.1 microfarad, 250,000 amplification. Note marked diphasicity of superimposed potentials.

2. Electroneurogram from same preparation after killing 4 mm. under grid electrode. Note simplification of record.

3. Electroneurogram after killing further toward ground electrode, 6 mm. live nerve now left. Note single sustained potential with a few superimposed short potentials.

4. Electroneurogram after further killing so that only 3 mm. of live nerve left under grid electrode.

multipolar cells exist alone they do not give rise to such long potentials on activation by other cells. They apparently also do not possess spontaneous activity. It is possible, in the preparations where more than one unipolar cell was found and where the sustained negative potential appeared simple, that not all the unipolar cells present were active. This is borne out by the effect of adding strychnine to certain of such preparations. Under such circumstances a simple potential may again become complex. The presumption is that strychnine excites some or all of the resting unipolar cells. In all the preparations crushing of the nerve could presumably have been carried farther and in many activity in a smaller number of cells might then have been shown to be associated with the sustained potential. The experiments were stopped to avoid destruction of the preparation for histological study. A more detailed study of the histology of the median nerve cord and of the nerve endings in the heart muscle is now being carried out.

Action of strychnine, acetylcholine, adrenalin and cocain on the complex activity potential and the sustained negative potential. When strychnine sulphate 1-2000 is diffusely applied to the median nerve cord the first effect is to increase the amplitude and frequency of the positive and negative phases of the potential complex, to increase its total duration and to increase the frequency of the spontaneous responses (fig. 4). The period of excitation is followed by a gradual depression so that in 15 to 30 minutes all spontaneous activity ceases. As the depression develops, it manifests itself by a slowing of the rate of total response, a lowering of the amplitude and frequency of the positive and negative phases of the potential complex and a decrease in its total duration.

These results are readily understood when the action of a similar concentration of strychnine sulphate on the sustained negative potential is studied on a short segment. The first effect is to increase the rate of its occurrence, to increase its amplitude and total duration (fig. 4). The frequency and sometimes the amplitude of the superimposed axon potentials are increased. The increased amplitude of the superimposed axon potentials when present is considered to be the result of a more perfect synchronization of the axon responses. Similar effects on the axon would be effected by the application of a galvanic current of increasing duration and strength.

Acetylcholine (1-500) in sea water applied to the nerve cord as a whole generally results in a slowing of the frequency of the total response, a lowering and shortening of the total potential complex with a lowering and slowing of the frequency of the positive and negative potentials. In a few preparations there has been observed for a few minutes an excitatory effect similar to that produced by strychnine. The depression from acetylcholine in the concentration employed is not rapid, 15 or more minutes are frequently required to depress to extinction all spontaneous activity.

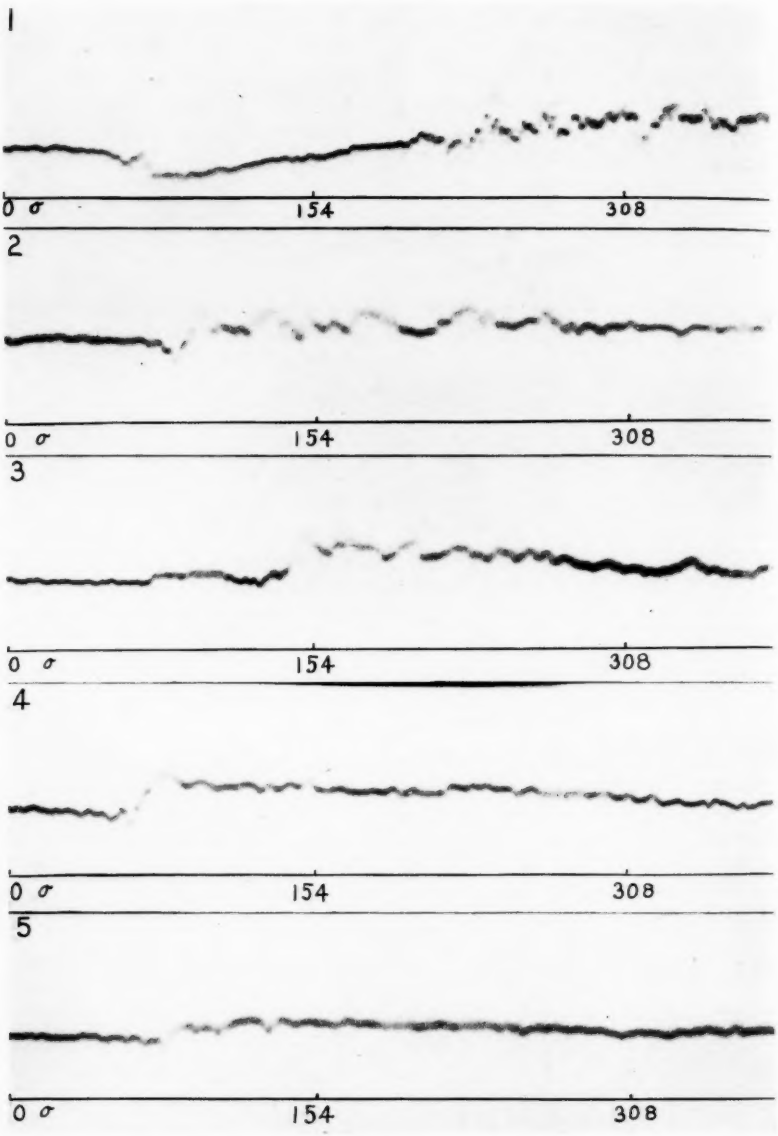

Fig. 6. 1. Electroneurogram from midportion of median cardiac nerve. Total nerve length 20 mm., interelectrode distance 11 mm., both electrodes on live nerve. Note unusual occurrence of a sustained potential with only a single superimposed

The effect of acetylcholine on the sustained negative potential is to decrease the frequency of its occurrence, to decrease its amplitude and to shorten its total duration (fig. 4). Superimposed axon potentials are decreased in frequency and often in amplitude. The depression of the rate of occurrence of the potential is relatively greater than is the depression of amplitude and duration of the potential. This is in contrast with the depressing action of cocain when the depression in rate of occurrence parallels approximately the depression in amplitude and duration.

Cocain (1–100) in sea water first acts as an excitant similar to strychnine. With the concentration employed depression soon sets in with changes similar to those produced by acetylcholine except for the difference noted above. Adrenalin hydrochloride (1–100,000) acts as an excitant with effects on the potential records similar to those produced by cocain as an excitant.

Discussion. The problem of determining the source of the sustained potential is a difficult one. The evidence that the sustained potential becomes more and more simple on shortening the interelectrode distance and is most free of all superimposed oscillations when the live tissue is not over 3 to 4 mm. in length indicates that it is not the result of a summation of parallel fiber potentials. The amplitude and duration of such a summated wave would be greatest when the interelectrode distance was considerable and the number of active elements therefore greater. Moreover, it would not be expected that such a summated potential would show any appreciable degree of constancy of amplitude and of duration when the superimposed potentials as recorded are so irregular in form and in amplitude. It seems evident that the oscillations superimposed on the sustained potential are fiber potentials, both because of their form which corresponds to that of established fiber potentials recorded from the nerve and after stimulation and also because of the ability to make them monophasic or almost so on killing under one electrode. Failure to make the monophasicity complete is presumed to be due to the presence of positive after potentials in fibers of this type.

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short potential until the base line is almost reached when other short potentials appear. Coupling condensers 2 microfarad, amplification 250,000.

2. Record with conditions same as in 1 after slight shifting of nerve on electrodes. Change of sign indicates the sustained potential is now recorded from the opposite electrode.

3. Electroneurogram after killing, 3 mm. of nerve under ground electrode. Note that superimposed short potentials tend to become monophasic.

4. Record after killing further toward grid electrode, 6 mm. live nerve left. Note further elimination of superimposed short potentials.

5. Record after further killing to leave 4 mm. of live nerve. Note some lowering of the sustained potential. This is not always the result on further killing. It is interpreted as an injury effect.

Assuming then that the sustained potential is a ganglion cell potential it is of interest to see to what extent the available evidence permits a designation of its source. There is first the evidence that the recorded potential from that part of the nerve cord corresponding to the anterior three cardiomeres when activated by impulses coming from the nerve cord more posteriorly does not show any of the sustained potentials. This anterior part of the nerve cord is not spontaneously active. Histologically it contains only cells of the multipolar type. It does not usually contain any of the large unipolar cells. The latter are concentrated in those cardiomere divisions which show a maximum degree of spontaneous activity. Our evidence shows that the presence of a single cell of this type, when associated with a few cells of the multipolar type, is adequate to produce a sustained potential. There is no correlation between the amplitude and duration of the sustained potential and the number of ganglion cells in the stretch of nerve giving rise to such a potential. The duration of the potential from a single cell with a few multipolar cells is as great as that recorded from preparations containing more than one unipolar cell and many more multipolar cells. While it is felt that the single cell itself is probably the source of such a sustained potential the evidence does not permit the statement as a demonstrated fact. Regardless of its source, the prolonged negative potential is associated with repetitive fiber responses. The stimulation effect on the fiber is regarded as a product of a potential gradient between axon and cell. The recovery period of the fiber is but a small fraction of the duration of the slow potential.

The spontaneously rhythmic ganglion cells of the heart cord appear to act in a manner similar to that of heart muscle which is itself rhythmic. Such rhythmic activity can be considered as a result of two processes, one the production of energy which, when it reaches a certain level, or threshold, induces spontaneous depolarization in the cell membrane, the other the depolarization of the cell membrane itself. Either of these processes might be altered by chemical or metabolic factors. Stimulation or inhibition of the energy building process might result in an increase or decrease in the rate of occurrence of ganglion cell depolarization. Alteration in the depolarization factor might result in changes in frequency and duration of its fiber responses. The latter, in turn, would determine the degree of the multipolar cell response. A nervous basis for the chronotropic and inotropic mechanisms of the heart of Limulus is thus suggested.

While the property of independent activity of an individual ganglion cell is not finally established by our results, they do demonstrate that the presence of a single cell of a certain type possibly in association with a small group of cells of a second type, in themselves incapable of spontaneous activity, may constitute a pacemaker mechanism. There is no reason

to believe that under the experimental conditions realized any excitant external to such a group was active in producing the cell activity. Such a cell can properly be designated as a pacemaker cell and it seems probable that it is the giant unipolar cell which serves such a function in cardiac nerve cord of Limulus. Such pacemaker cells are almost certainly present in other rhythmical centers such as the respiratory center. Adrian and Buytendyk's (1931) evidence of rhythmical activity in the gold fish brain with a rate corresponding to that of the animal's respiration adds support to this interpretation. Our findings in the Limulus heart cord correspond closely with those reported by Adrian (1931, loc. cit.) in his observations on potential changes in Dytiscus marginalis.

SUMMARY

Individual ganglion cells assumed to have pacemaker functions, or such cells in association with small groups of other cells in the median cardiac nerve of Limulus polyphemus show spontaneous activity when entirely separated from the body.

During such activity the single ganglion cell or such a cell in association with a small group of other cells, develops a sustained negative potential.

During this period of sustained negativity there is a repetitive nerve fiber response.

The sustained negative potential develops rather rapidly and then slowly subsides. There is no evidence of any appreciable positive after-potentials.

Activity in the fibers develops during the rising phase of the sustained potential and continues throughout the greater part of the period of negativity. The fiber response is most rapid during the height of the sustained potential and is slower as it subsides.

Strychnine, as an excitant, causes an increase in amplitude and duration of the sustained negative potential. The frequency of occurrence of the sustained negative potentials is increased as is the frequency of the fiber response during each period of sustained potential.

Acetylcholine as a depressant causes a slowing of the frequency of development of the sustained negativity and a lowering and shortening of the potential. There is a diminution in the frequency and duration of fiber response.

Adrenalin and cocain in concentrations causing increased excitability cause changes in the sustained negative potential similar to those produced by strychnine. As a depressant cocain decreases the amplitude of the sustained negative potential to a greater degree than does acetylcholine for a corresponding degree of slowing of the frequency of its occurrence.

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OBSERVATIONS ON THE RESPONSE OF THE SPLEEN TO THE INTRAVENOUS INJECTION OF CERTAIN SECRETIN PREPARATIONS, ACETYL CHOLINE AND HISTAMINE

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We were interested in the effect of certain secretin preparations, or duodenal extracts, on the spleen because various reports in the literature indicate that a definite, but transient, erythrocytosis occurs after the injection of secretin preparations (1, 2, 3), and because a constriction of the spleen is believed to cause a temporary erythrocytosis (4, 5). Krzywanek (6), moreover, has suggested that the spleen plays a part in this phenomenon, basing his opinion on findings that the increase in erythrocytes per cubic millimeter after the injection of a secretin preparation was less in splenectomized than in normal dogs. However, he did not use a vasodilatin-free secretin.

As we found that a certain secretin preparation (S_1, vide infra) quite uniformly caused the spleen to constrict, we studied the effect of acetylcholine and histamine on the splenic volume, thinking that the response might possibly be due to minute traces of these substances. We were interested particularly in ascertaining the effect of acetyl choline on the spleen because Frederieq (7), studying the response of the excised organ of the dog to drugs and nerve stimulation, suggested that it may receive a parasympathetic innervation. We were unable to find a report in the literature regarding the effect of histamine on the splenic volume of the dog or on splenic strips.

METHODS. To determine directly the effect of certain secretin preparations, or duodenal extracts, on the volume of the spleen, we anesthetized a number of dogs (15–30 lbs. in weight), using sodium barbital or sodium pentobarbital, and ether. The spleen was enclosed in a plethysmograph and tracings were made before and after the intravenous injection of the substances studied. A blood pressure record was made simultaneously. The preparations used are designated S_1, Aniline-filtrate, and Aniline-precipitate by Ivy et al., and are described in their publications (8, 9, 10).

S_1 is a preparation which contains both secretin (active in 0.25–0.5 mgm.

doses) and cholecystokinin (active in 2.5–3 mgm. doses). The aniline-filtrate preparation contains chiefly secretin and is active in 0.05–0.025

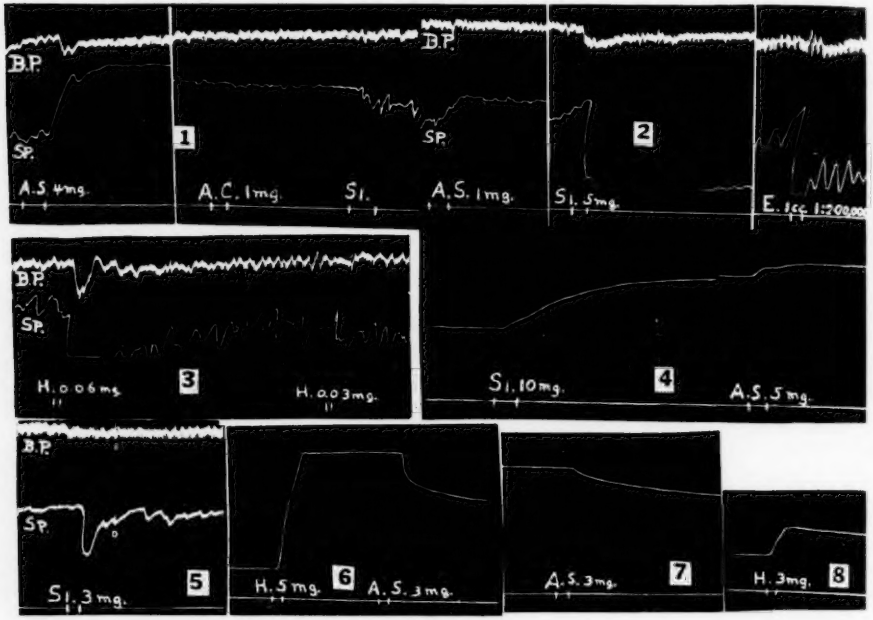

Fig. 1. *B. P.*, blood pressure; *Sp*, spleen volume; *Up*, dilatation; *Down*, constriction; *A. S.*, atropine sulphate, *A. C.*, acetyl choline; *E*, epinephrine; *H*, histamine.

Tracing 1 shows dilatation after atropine and the failure of *A. C.* to work 10 minutes later; S_1, however, caused a constriction 5 minutes after the *A. C.*

Tracing 2 shows dilatation after atropine followed by a constriction of the spleen 5 minutes later from S_1 and then a few minutes later epinephrine 1 cc. 1:200,000 caused an analogous constriction except that it was of shorter duration.

Tracing 3 shows constriction of the spleen from 0.06 mgm. of histamine together with a drop in blood pressure and the ineffectiveness of 0.03 mgm. to lower the blood pressure or to constrict the spleen.

Tracing 4 shows the contraction of a splenic strip brought about by 10 mgm. of S_1 and the ineffectiveness of atropine to inhibit the action; in fact, atropine appeared to cause contraction.

Tracing 5 shows a typical response of the spleen to 3 mgm. of S_1.

Tracing 6 shows a contraction of a splenic strip from 5 mgm. of histamine, and a relaxation induced by 3 mgm. of atropine.

Tracing 7 shows a typical relaxation of a splenic strip from atropine.

Tracing 8 shows a typical contraction of a splenic strip from 3 mgm. of histamine.

mgm. doses. It stimulates the gall bladder slightly in 1 mgm. doses. The aniline-precipitate preparation contains only traces of secretin, but contains some active cholecystokinin (active in 5 mgm. doses).

RESULTS. *S_1 preparation of "secretin."* Twenty out of thirty dogs manifested a definite constriction of the spleen after the injection of 5 mgm. of S_1 preparation. The minimum effective dose was 2 mgm., or four threshold doses of secretin. A constriction of the spleen was quite uniformly obtained with successive injections in responsive animals; in twenty reactive animals, we recorded forty-five constrictor responses in sixty-three trials. A dilator response was not observed. The result of a typical constriction of the spleen is shown in figure 1, tr. 5. In volume the constriction obtained using 3 to 5 mgm. of S_1, generally amounted to from 2 to 3 cc., occasionally more. The degree of constriction obtained was comparable to that observed after the injection of 1 cc. of a 1:200,000 solution of epinephrine. The constriction was not prevented by atropine sulphate (4 mgm.); but occasionally during a post-injection-atropine period of about ten minutes the constriction appeared to be reduced, after which period the usual constrictor response was obtained. From 1 to 4 mgm. of atropine sulphate were used (fig. 1, tr. 1 and 2).

The aniline-filtrate preparation of "secretin." This preparation was used as we believe it to be almost pure secretin. Although this preparation was injected eighteen times into eight dogs in doses as large as 1 mgm. (20 plus doses of secretin), a definite constriction of the spleen did not occur. In five tests a very slight decrease in volume resulted; in five others an increase in the rhythmical changes in volume occurred; and in eight trials no change or a very slight change in volume occurred. These results showed that the substance in the S_1 preparation which caused the constriction of the spleen was either in the aniline-precipitate or had been destroyed by the chemical treatment. These results, also, showed that the substance in S_1 causing constriction of the spleen is not secretin.

The aniline-precipitate preparation. The aniline precipitate was injected twenty-four times into nine dogs in doses as large as 10 mgm. In no instance was a definite change in the volume of the spleen observed. In two cases, one in which a dose of 10 mgm. and the other in which 5 mgm. were injected, a slight constriction occurred each of which could be interpreted as a spontaneous variation.

The failure of the aniline filtrate and precipitate to cause a definite constriction shows that the aniline method of concentrating secretin inactivates the substance in duodenal extract which causes constriction of the spleen.

Not being able to concentrate the splenic constricting substance in S_1, we decided to ascertain if the constriction might be due to traces of acetyl choline or histamine. These substances, if in the S_1 preparation, must be present only in minute traces because the systemic blood pressure was never more than slightly influenced by the injection of S_1.

Acetyl choline. One milligram of acetyl choline caused a constriction of the spleen. The amount of constriction compared favorably to that

obtained with from 3 to 5 mgm. of S_1. The constriction persisted considerably longer than the fall in blood pressure which indicated a true contraction of the spleen. This interpretation is supported by the observation that in two instances during an atropine recovery period the spleen showed constriction on the injection of acetyl choline without a change in blood pressure. We always obtained a constriction of the spleen with acetyl choline, and are at a loss to explain the variable responses observed by Hunt (11).

Atropine sulphate. Atropine, contrary to the results of Krafka, McCrea and Vogt (12) on the excised spleen of the hog and calf, in 1 mgm. doses caused a slight, and in 4 mgm. doses a definite dilatation of the spleen (fig. 1, tr. 1 and 2). Atropine sulphate in 1 mgm. doses abolished the constrictor action of acetyl choline for about ten minutes; in 4 mgm. doses atropine abolished the constrictor action for about fifty minutes. However, in one dog a typical constriction was obtained 20 minutes after 4 mgm. of atropine and before the typical effect of acetyl choline on the blood pressure had returned.

Histamine. Histamine in doses which definitely reduced the blood pressure caused a contraction of the spleen (fig. 1, tr. 3). It was found that 0.0625 mgm. (10–15 kilo dogs), which caused a fall in blood pressure of 24 mm. of Hg, resulted in a constriction which was approximately equivalent to that caused by 3 to 5 mgm. of S_1, which outlasted the period in the fall in blood pressure by from 5 to 10 minutes. A dose which would cause only a 10 to 15 mm. fall in pressure would cause a definite constriction lasting 5 or 10 minutes. A dose, such as 0.0321 mgm. (15–18 kilo dog), which produced no fall in blood pressure, caused little or no constriction. Thus it follows that the constriction of the spleen after histamine administration is related directly to the fall in blood pressure.

Use of splenic strips. In order to confirm our results on the intact animal when using the various substances mentioned above, we determined the effect of these drugs on strips of fresh spleen of the dog suspended in a bath.

Methods. The strips were approximately 5 cm. in length and included the more muscular part of the spleen, that is, the inner part of the capsule. The bath in which the strips were immersed was of a composition recommended by Sollmann and Rademaekers (NaCl 0.9 per cent; KCl 0.042 per cent; $CaCl_2$ 0.012 per cent; $NaHCO_3$ 0.03 per cent; glucose 0.1 per cent). The pH of such a solution is 7.8. It was oxygenated by a stream of air, which was washed with a 0.1 per cent solution of sodium bicarbonate. The temperature of the bath was held at 37.5°C. The volume of the bath was approximately 100 cc. In many instances it was necessary to use a fresh strip for each test as a spontaneous return to normal after a strong contraction is so long delayed that the further use of the strip is impracticable.

Results. Acetyl choline. Fredericq (7) and Vairel (13) reported that acetyl choline will cause a contraction of a splenic strip and that epinephrine will induce an additional contraction. Working on splenic strips in a bath as indicated above, we obtained a contraction using acetyl choline and an additional contraction with epinephrine. After adding 1 mgm. of atropine sulphate to the bath, 3 mgm. of acetyl choline were ineffective.

Atropine sulphate. Atropine sulphate when added to the bath caused a prolonged relaxation of the splenic strip. Doses of 3 to 5 mgm. appeared to give maximal effects. Typical results are shown in figure 1, tr. 6 and 7.

Histamine. The results from histamine were somewhat variable. In the majority of cases the strip contracted (fig. 1, tr. 6 and 8), but in certain instances a slight relaxation occurred.

S_1 preparation of secretin. S_1 in dose of 10 to 20 mgm. gave a marked contraction of the splenic strip (fig. 1, tr. 4). From the tracing it is clear that atropine does not counteract the effect of S_1; in fact, atropine either causes a contraction or sensitizes the strip to S_1. This odd phenomenon was not studied further because the supply of S_1 was limited.

Discussion. Studying the action of histamine on the spleen of the cat, Dale and Laidlaw (14) found that it causes a constriction which did not last as long as the fall in blood pressure. They thought that histamine caused an active contraction of the musculature of the spleen and that this was a more important factor than the fall in blood pressure. However, they did not use doses of histamine which have no effect on systemic blood pressure, as we did, and our results show that to obtain constriction of the spleen with histamine a dose which causes some fall in blood pressure must be employed. Hence, the constriction of the spleen in situ caused by histamine is either a response caused by a fall in blood pressure or the excitatory dose of the chemical for the splenic musculature is identical with the vasodepressor dose. Relatively large doses of histamine caused, as a rule, a contraction of splenic strips, although the effect was variable. It is possible that *in situ* histamine reduces the volume of the spleen by causing its musculature to contract, but in ordinary doses this seems unlikely. Regardless of the mechanism by which histamine causes constriction of the spleen, it is evident from our results that the constrictor substance in the S_1 preparation is not histamine, which was the point of chief interest to us.

Our observations on the effect of acetyl choline and atropine on the volume of the spleen and on splenic strips corroborate the observations of Fredericq (7) on the effect of these drugs on the isolated spleen. Our *in vivo* and Fredericq's *in vitro* results, which we confirmed, show that the splenic musculature is responsive to parasympathomimetic as well as sympathomimetic drugs. The fact that we occasionally observed recovery of the spleen from the atropine block of acetyl choline before the blood

pressure effect returned indicates that the splenic musculature is somewhat analogous to that of the intestine in being refractory to atropine.

It is clear that neither histamine nor acetyl acholine is the spleno-constrictor substance present in our S_1 preparation for the following reasons: *a*, the injection of S_1 frequently causes constriction without the slightest change in blood pressure and when a change in blood pressure does occur it is so slight as not to explain the unproportionate or marked constriction of the spleen; *b*, the constriction of the spleen caused by histamine *in vivo* is directly related to the change in systemic blood pressure because a constriction of the spleen was never obtained by a dose of histamine too small to cause a change in blood pressure; *c*, the constrictor effect of acetyl choline is counteracted by atropine for from ten to fifty minutes, while the constrictor action of S_1 is counteracted at no time by atropine. The same is true for splenic strips. We have no explanation to offer for the fact that some dogs appear to be refractory to the S_1 preparation, whereas they are not refractory to acetyl choline, histamine, or epinephrine. The musculature of the duodenum, though quite uniformly caused to contract by this preparation (S_1) is also refractory in some dogs (15).

Since a preparation of secretin can be made which in relatively large doses has no definite constrictor action on the spleen, secretin is not the spleno-constrictor substance in duodenal extracts. We suspect that the spleno-constrictor substance in our S_1 preparation from duodenal mucosa is the same substance observed frequently to cause contraction of the duodenum and intestine in the cholecystokinin preparation used by Sandblom, Voegtlin, and Ivy (15) and practically absent from the cholecystokinin preparation used by Lueth, Ivy and Kloster (16). For this reason and because our aniline precipitate contains cholecystokinin, we believe, at present, that the spleno-constrictor substance is not cholecystokinin. Yet, a variation in the threshold irritability of the gall bladder, duodenal and splenic musculature may explain our results. We suspect, however, that an excitant of the intestinal and splenic musculature other than cholecystokinin, acetyl choline and histamine will be isolated at some future date from the duodenal mucosa (17). To date we have had no success in preparing such a motor excitant free from both secretin and cholecystokinin.

CONCLUSIONS

1. A spleno-constrictor substance is present in certain extracts of the duodenal mucosa.

2. The spleno-constrictor substance is not acetyl choline, histamine, or secretin and probably is not cholecystokinin.

3. We have been unable to concentrate by fractionation the spleno-constrictor substance, but point out in the discussion that it is probably

identical with the substance in the duodenal extract which augments intestinal motility as referred to by Sandblom, Voegtlin and Ivy.

4. Acetyl choline causes contraction of the spleen *in vivo* and contraction of strips of the spleen. Its action is antagonized by atropine.

5. Certain duodenal extracts cause contraction of splenic strips which is not antagonized by atropine.

6. Histamine causes contraction of the spleen *in vivo* in doses that affect blood pressure and relatively large doses of histamine are required to cause contraction of splenic strips.

7. Atropine causes relaxation of the spleen *in vivo* and relaxation of splenic strips.

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STUDY OF DEPTH TEMPERATURES IN ARTIFICIAL FEVERS AND COOLING AIR CHAMBERS WITH ESPECIAL REFERENCE TO COOLING EFFECT OF THE CIRCULATING BLOOD

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Studies of deep internal temperature in man have been made since the time of Becquerel and Breschet (1), who in 1835 first used electrical thermocouples for this purpose. Recent investigations in this field have been made by Lefevre (2), Zondek (3), Bazett and McGlone (4), Foged (5) and Wright and Johnson (6). Of early animal experimentation, Claude Bernard (7), Liebig (8) and Colin (9) did the most extensive work demonstrating the heating of the blood by the somatic muscles, the liver and the intestines, and cooling of the blood by surface exposure.

It is recognized that heat gradients exist in man in zones from the skin surface to various depths and that these gradients vary under circumstances of formation or loss of heat throughout the body and may vary in different portions of the same individual's body at the same time. As suggested by Bazett (10) and Deighton (11) but, to my knowledge not yet proved, the gradient from the skin to deeper tissues may be reversed on recovery from cold or onset of fever, and Bazett and McGlone (4) have shown that the deep skin layer may be cooler than the superficial one.

It is the purpose of this paper 1, to show that such gradient reversals are exhibited in artificial fever and occasionally at normal temperatures; 2, to confirm the observations that cubital venous blood is usually cooler than most tissues, and that gradient changes are sudden from skin to the generally cool subcutaneous tissue and warm striated muscle; and 3, to demonstrate that, whereas, blood may serve to heat tissues on returning from a heated skin or active muscles and tissues, it may often be an important cooling agent for body tissues. Especially when skin cooling is excluded, is it apparent that much heat is lost from the blood in the respiratory tract.

TECHNIQUE. The apparatus used for studying such temperatures in the human body was a copper-cupron (a copper nickel alloy) thermocouple junction imbedded in a bakelite collar in the tip of a no. 22 gauge rustless steel needle, 10 cm. in length, with the customary circuit of galvanometer

and thermocouple connection in a water bath of known temperature. The apparatus was calibrated and thermocouple readings checked by standardized clinical thermometers in the oral and rectal readings. The readings could be made in less than five seconds and the needle apparatus showed no demonstrable tendency to conduct heat away from the thermocouple junction in this period. In taking intravenous temperature the thermocouple needle was inserted into a no. 18 gauge steel needle that was placed in the vein in such a manner as to project about 2 mm. beyond the tip of the latter needle.[1]

The entire arm, except at the point of puncture, was kept warmly but not tightly covered when intravenous blood temperature readings were made in the cubital vein, and was not removed from the blankets when observing temperatures in artificial fever cases. The normal men were put in warm beds for $\frac{3}{4}$ to 1 hour before temperature readings were made. Four adult men with normal temperatures were observed, seven with fever produced by swathing in blankets and rubber sheets by the Epstein and Cohen technique (12), two men with fever produced by both electric light cabinet and blanket swathing and one by intravenous administration of typhoid-vaccine. The cooling effect of inclosing the head and neck in a tent in which supercooled air was circulated was studied in two men with normal temperatures and one with "blanket pack" fever, and the cooling influence of cold sponge baths was studied on two of the artificial fever cases.

RESULTS. *General normal temperature gradients.* Typical observations are illustrated in table 1. The relation of the various normal temperatures agreed in general with the findings of Wright and Johnson (6), Bazett and McGlone (4) and others. In one man (Mr. V., table 1) whose layer of

[1] I am indebted to W. W. Salisbury for the construction and testing of the thermocouple needles used. With 10°C. difference in the temperature between the measured point and the room, his estimation of heat loss in the no. 42 gauge copper wire of the thermocouple was 4.6×10^{-6} calories per second and of the no. 42 gauge cupron wire was 4.5×10^{-7} calories per second. Assuming the heat conductivity of water as 0.0014 and that blood approximates this, a maximum error of 0.065°C. may occur but much less than that if the blood is in motion. Assuming that human fat approaches the heat conductivity of castor oil, namely, 0.000425, the maximum error in the subcutaneous tissue reading could be 0.25°C. The conductivity of muscle probably lies between the above two estimates.

The bakelite sheath is an excellent heat insulator and with at least 1.5 cm. of the needle buried in tissue, little significant error can be attributed to heat loss from these sources.

The galvanometer reading actually became stationary within one second in all observations except those of the skin, in which the above errors may have been present, and in the intravenous readings in certain infrequent instances when the skin was temporarily uncovered to place the thermocouple and then recovered to attain its previous temperature.

superficial fat was unusually thin, temperatures were much lower in all the tissues of the exposed arm than in the covered thigh. Mark and later Wieland (cit. Deighton, 11) have previously explained such findings as evidence of the insulating property of fat. Intramuscular temperature exceeded the rectal temperature in only one of seven cases but exceeded or equalled the oral temperature in three cases. The intravenous blood temperature was lower than all but the surface skin temperature in five out of seven cases, excluding the deltoid region temperature readings in Mr. V. (table 1). In the other two cases only the temperature of the subcutaneous tissue as well as the skin was lower than the intravenous readings.

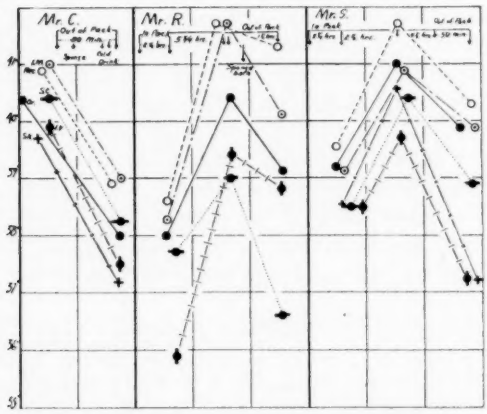

Fig. 1. Temperature relations in cooling by surface sponging in artificial fever cases. *I-M*, *S-C*, *I-C*, *Sk*, or *Cut* refer respectively to intramuscular, subcutaneous, intracutaneous, and surface skin temperature readings of the same thigh region and "I-V" to intravenous blood (median basilic vein). Temperature readings all by the thermocouple needle method. *Or* and *Rec* indicate oral temperature and rectal temperature respectively as taken by clinical mercury thermometers. Such readings were frequently checked against thermocouple determinations.

Three out of the seven cases with normal temperatures exhibited skin surface temperatures equalling or exceeding those of the subcutaneous tissue (table 1—case of Mr. A.—quadriceps-femoris region; Mr. V.—deltoid region) (fig. 1—Mr. C.—buttocks region). Insofar as these cases did not represent general temperature changes when the observations were made, it is difficult to account for this phenomenon otherwise than by loss of heat from the surface elsewhere than in the region studied, or by a general cooling effect of arterial blood. The latter was assumed to be the sole operative mechanism in the "blanket pack" fever cases.

Temperature gradients with artificial fever. This reversal of skin-subcutaneous tissue gradient was the common finding in cases of "blanket pack" fever production (four out of six observations) and was sometimes associated with a wider separation between temperatures of the venous blood and the other tissues; the former rising more slowly than the rectal, oral or intramuscular temperatures (fig. 2). Herein is demonstrated an instance in which the blood assumes the chief cooling function of the body, since with no evaporation possible, little heat can be lost from the skin. The temperature in the deep layers of the blankets generally approached the oral temperature and exceeded the skin temperature.

TABLE 1

Comparative temperatures of individuals without fever (Centigrade)

	RECTAL	ORAL	INTRAMUSCULAR		SUBCUTANEOUS		SKIN SURFACE		INTRACUTANEOUS		INTRAVENOUS
			Deltoid	Thigh	Arm	Thigh	Arm	Thigh	Arm	Thigh	
Mr. A., well-nourished	37.7	37	36.4	36.45	34.9	34.85	34.3	34.9	31.7	33.8	35.6
Mr. V., mal-nourished	37.4	36.6	35.75	36.9	32.9	36.3	33.6	35.5	31.3	34.3	34.4
							Skin neck				
Mr. R.	37.1	36.6		37.7		36	35	35	32	32.5	35

	RECTAL		ORAL		
	Superficial	Deep			
Mr. D., hepatic cirrhosis	37.5	36.8	36.8	Abdominal wall Ascitic fluid Intra-peritoneal space	37.4

Another good example of this reversal of the gradient of the skin and subcutaneous tissue in which the rôle of the blood is more clearly evident, occurs in case 3, Mr. V., figure 2. In this case the patient was lightly clothed and exposed to the hot air in the electric cabinet for a sufficiently prolonged period to produce fever. The skin still remained cooler than the other tissues, as evaporation could still take place. The blood, however, was carried away from the surface appreciably warmed, its temperature exceeding even that of the subcutaneous tissues. The normal gradient was still maintained with this exception. On removing the cabinet and wrapping the patient in blankets the reversal of the gradient occurred. The skin was no longer an effective means for heat loss and became warmer

than the subcutaneous tissue. Increased metabolism of the skin itself may partially account for its high temperature under such circumstances. The blood temperature actually fell since it then became an agent of cooling from within instead of heating from without.

Temperatures recorded deep in the skin were always the lowest observed in any patient but were slow to come to equilibrium. Such low readings may be due to shutting off adjacent blood supply by pressure of the needle in the firm tissue layers and may not, therefore, represent a true state. Deep skin temperatures, low relative to that in other tissues, likewise have

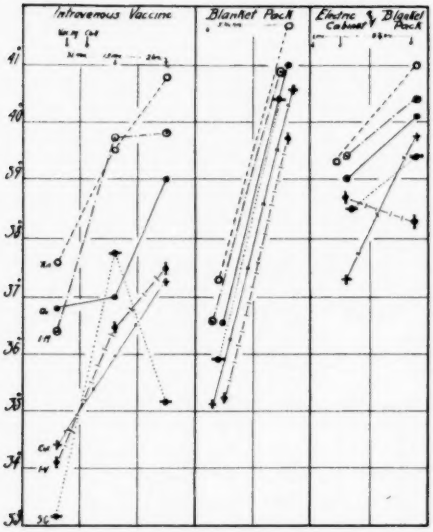

Fig. 2

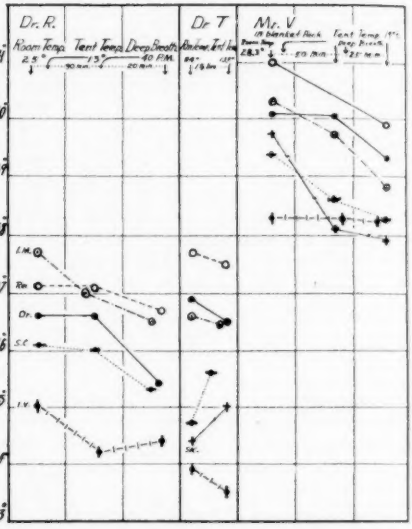

Fig. 3

Fig. 2. Temperature relations in artificial fevers. Symbols as in figure 1.

Fig. 3. Temperature relations in artificial cooling by iced air. Symbols as in figure 1.

been reported by Bazett and McGlone (4). Intramuscular temperatures generally rose somewhat more rapidly in the "blanket pack" fever cases than oral or rectal, exceeding the latter temperature at the height of the fever in four out of six cases. (Fig. 1—cases 1 and 2.) The prompt fall of intramuscular temperatures upon release from the "blanket pack," equalling the rate of subcutaneous tissue temperature drop, suggests that the mechanism of fever production was probably purely one of retention of heat and not excess formation in the muscles or other active tissues. If such excess heat formation existed, some lag of the temperature fall curve might be expected.

The behavior of intramuscular temperature in the vaccine fever case (case 1, fig. 2), is interesting because it rises rapidly with the chill as expected, but fails to continue upward with the rising oral and rectal readings. One may deduct that surface heat loss, as evidenced by the falling subcutaneous temperature, equals the heat formation in the muscle which is obviously less in degree than with the contractions during the chill. It is then assumed that excess heat formation proceeds at a more even rate in the abdominal organs and because of their depth the generated heat is better retained than in the superficial muscles. The constant rise of venous blood temperature suggests that the elevated general body temperature cannot be easily explained in the presence of a loss in subcutaneous tissue heat except as previously discussed.

Temperature gradients with general body cooling. Certain mechanisms of heat loss are illustrated in two cases with surface sponging at the peak of the fever (fig. 1). All temperatures fell uniformly in the first case but the intravenous temperature, proportionately to other temperatures, fell more slowly in the second. This behavior resembles the level state of the intravenous temperature in figure 3—Mr. V.— when other tissues were cooling, and illustrates how the blood may act as a temperature stabilizer. Its high specific heat tends to maintain a gradient in a warmer or cooler region, permitting the removal or supply of heat without the blood rapidly approaching the temperature of its environment.

It has been previously illustrated how blood serves as a cooling agent when the skin is eliminated as the chief source of heat loss. The respiratory rates of these patients with artificial fever are commonly over 25 per minute which acceleration would tend to increase the heat dissipating function of the respiratory tract. If this mechanism alone were adequate it is apparent that little fever would be produced. An index of this adequacy is the slow rise of the general body temperature (oral and rectal) and its maintenance at a maximum of over 40°C. orally for 3 to 5 hours with little further rise in spite of continuance of the pack. Thus one may suggest that in man the respiratory tract has great potential means for heat loss, at least under special circumstances.

Tigerstedt (13) estimated that 5 per cent of all the heat loss normally occurs in warming air and ingested fluids and food and 10 per cent in release of CO_2 in the lungs and saturating inspired air with water (to 95± per cent). This estimate must be erroneously low under the circumstances of unusually cold or dry air, rapid or deep respiration, or fever. Barach (14) and others have described the fall in temperature in pneumonia patients placed in cooled oxygen tents. Exclusive of the rapid respiration rate of such patients, the continued inspiration of cool, dried air in such apparatus operates to drop the general body temperature. This is illustrated by the following observations.

Figure 3 illustrates two series of temperature observations of normal individuals (Drs. R. and T.) before and after inspiring iced air in a bed tent. At first their respiratory rates were 18 per minute and later 32 to 40 per minute. The fall of all tissue temperatures in the case of Dr. R. was definite, especially after rapid deep breathing. As previously stated, the tent was placed over the head and neck and the remainder of the body remained at room temperature. The ordinary reaction to local cold (Bazett) is a general contraction of skin capillaries which tends to retain heat and not drop deep tissue temperature. It may be assumed that the blood was overcooled leaving the lungs, and served as a general cooling agent to all body tissues. This is strongly suggested in the observations on Dr. T. (fig. 3), since the skin and subcutaneous tissue actually were warmer at the end of the experiment than at the beginning. This was probably caused by the tissues approaching equilibrium with the higher room and bed temperatures on recent return of the individual from exposure to the lower outdoor temperature.

The temperature drop in various tissues in the artificial fever case in which a similar iced air tent was used was striking. However, the stability of the venous blood temperature fails to suggest unusual arterial blood cooling. It thus resembles the observations made on the cooling of fever patients by surface sponging and recalls the comment made on the high specific heat of blood. Some of the heat loss in these iced air tent experiments must take place from the face as well as from the respiratory tract and it is admitted that this may be an appreciable quantity.

SUMMARY AND CONCLUSION

Temperature determinations of skin surfaces, deep skin, subcutaneous tissue, muscles and blood in the precubital veins were made on normal individuals and on normally afebrile patients undergoing artificial fever treatments. Such observations were made under normal circumstances, at various stages of the fever production by 1, intravenous vaccine; 2, the "blanket pack" method alone, and 3, the "blanket pack" preceded by heating in an electric light cabinet. Observations were likewise made on such individuals during cooling by surface sponging and by inspiring iced air. Individuals with normal temperature were likewise studied under the influence of cooled air inhalation.

The recognized gradients between the deep tissue and surface skin were observed with the exception that occasionally the skin was warmer than the subcutaneous tissue under normal circumstances. This phenomenon generally occurred during artificial fever with the "blanket pack" method. The behavior of the intravenous blood temperature under various circumstances leads to the conclusion that the blood may serve as an important cooling agent to the general body tissues, losing more heat in the respiratory tract than has been believed heretofore.

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